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HEPATOCARCINOGENESIS IN LABORATORY RODENTS: RELEVANCE FOR MAN

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FOREWORD

The ECETOC Monographs nos.2 and 3, published in Sept. 1980 and Jan. 1982 respectively, dealt with the assessment of risk from occupational chemical carcinogens in general. These have been well received, and over 2,000 copies of each have now been issued.

In this current Monograph a particular aspect of human carcinogenicity and risk assessment is examined, ie. the relevance to man of liver cancers experimentally-induced in rodents by exposure to chemicals. This is important because although cancer of the liver is rare in humans relative to cancer in certain other organs it is not-infrequently observed in studies on rodents, and it is thus vital to make the best estimate of which of the chemicals involved pose a risk to man. It is hoped that this Monograph will assist all those in industry, trade unions, government, international organisations and universities who are responsible for, or concerned about, the protection of people from carcinogens.

It is with pleasure that I present this Monograph to its readers.

H.J. Heller
Chairman, ECETOC

EXECUTIVE SUMMARY

Studies to determine the carcinogenic potential of a chemical are often conducted in animals. The results of such studies constitute an important part of the evidence from which decisions are made on the likelihood of the chemical causing cancer in man under appropriate exposure conditions.

Many such carcinogenicity studies have been conducted in rats and mice over the last three decades and many of the chemicals tested caused cancer of the animals'liver; indeed the liver was frequently the only organ affected. Since cancer of the liver in man is a relatively uncommon disease, particularly in the Western world, it is important to establish whether the occurrence of cancer in rodent liver means that humans will be similarly affected if exposed to the chemical. This problem is addressed in this Monograph.

Unlike humans, a relatively high proportion of the commonly used laboratory rodents (rat and mouse) can develop liver cancer in the absence of deliberate exposure to defined chemicals. In mice the incidence can reach 100%. In rats, data are emerging which show that the background incidence in this species may also be quite significant (up to 29% in a recent study). In both species the incidence varies considerably in different strains and at different times in the same strain. In addition, the animals' diet (particularly the calorie intake and the fat and protein content) and the degree of freedom of the animals from natural parasites and other organisms (so-called "microbiological status") can considerably alter the background incidence of liver cancer.The first essential step is to establish the probability that the chemical caused liver cancer in rodents by virtue of its own action (or that of its metabolites) on important cell constituents.The second step is to examine the mechanisms by which the cancers developed. Two broad types of mechanism are considered. In the first (genotoxic) mechanism, the chemical is thought to act by altering the structure of the genetic material of the cell in such a way that it is converted from a normal to a cancerous cell. In the second (non-genotoxic) mechanism, the cancer develops from a non-cancerous lesion in a tissue or organ. This lesion, which would normally undergo healing or would fail to develop, becomes cancerous when it is prevented from healing by continued exposure to the chemical at high levels over long periods. Substances which produce cancer by this type of mechanism are generally found not to damage the genetic material directly.

The distinction between the two mechanisms is important since, in theory, a single molecule of a genotoxic carcinogen could lead to a cancer so that it would be impossible to define a safe level of exposure. Many scientists however agree that a safe level probably exists for genotoxic carcinogens but that insufficient information is currently available to define it for any of them. On the other hand, the safe level of a non-genotoxic carcinogen could be expected to be that which does not produce the type of lesion from which cancer develops.

The class of carcinogens into which any particular substance falls can be investigated by a series of studies on a) its ability to react with, or alter, the genetic material in such a way that cancer is likely to develop, and b) the development and progress of liver changes known to be associated with the development of cancer caused by non-genotoxic compounds. A flow chart (Tables 3 and 4) has been designed to assist in deciding the sequence in which such investigations might be carried out. This is intended as a guide to, rather than a rigid schedule of, the tests necessary to examine a new material or a substance which previous work has suggested may have cancerous effects in the liver of rodents. Only from the results of such studies can an assessment be made of the probability that a chemical will cause cancer in man.

Many of the concepts and methods used to investigate the relevance to man of rodent liver tumours are equally applicable to substances causing cancer in other organs and tissues.

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A. PREFACE

Long-term bioassays have shown that when rodents are fed on diets containing certain chemicals, or are otherwise exposed to them, benign and/or malignant tumours of liver cell origin may arise. Some strains,particularly of mice, are especially sensitive to the development of such tumours. Consequently the question then arises : what is the significance of these findings for man ? ECETOC, being aware of the importance of this question, set up a Task Force with the following Terms of Reference :

"To lay down the sequence of steps to be taken to assess the relevance to man of the occurrence of liver tumours in rodents which were exposed to a well-defined compound".

This Monograph aims to provide toxicologists with advice on the steps to be taken when investigating :

a) an increased incidence of hepatic tumours in rodents exposed to a chemical;

b) the assessment of a previously untested chemical for hepatocarcinogenicity.

The problems posed by the increased incidence of liver tumours in rodents exposed to certain chemicals need to be considered in an appropriately broad context.

"Hepatocarcinogenicity" (this term, as used throughout the report, encompasses the production of benign as well as malignant tumours of liver cell origin) is to be seen as a sub-section of the general subject, "Carcinogenicity". There would be little value in establishing that a potent carcinogen for, say, the brain was not a carcinogen for the liver. Inevitably, some of the discussion in this report is as relevant to problems in carcinogenesis generally as to hepatocarcinogenesis in particular.

There are good reasons for directing attention specifically to the liver as a potential target for carcinogenicity. Most chemicals absorbed from the gastro-intestinal tract reach the liver via the portal vein. For such substances, the liver serves as the first and major organ of metabolism and detoxification. Depending on dosé, some compounds are wholly metabolised on the first pass through the liver. The liver also plays a major metabolic role for lipids absorbed from the gut in chyle and for substances absorbed by other routes (e.g. the lungs, the skin). The fact that metabolites of absorbed compounds may be returned to the gastro-intestinal tract via the bile (the enterohepatic pathway) is also a unique feature of the liver in relation to carcinogenesis since the bacterial flora of the gut may further metabolise substances excreted in bile so

that secondary metabolites present in the gut, including some that are potentially hepatocarcinogenic, may reach the liver by absorption.

The subject of hepatocarcinogenicity is very complex and has been the subject of much debate and numerous reviews. It is not the purpose of this Monograph simply to review the whole complex situation yet again. On the contrary its aim is to discuss two questions of great practical importance not only for those industries who produce, handle or sell chemicals such as drugs, pesticides, manufacturing aids etc., but also for all human kind.
The two questions are :

- How should the finding of an increased incidence of liver tumours following exposure of laboratory animals to a test chemical be interpreted in terms of possible cancer risk (in the liver or at other sites) for man ?

- How may it be established whether a new chemical carries such a risk for humans?

In relation to the first question, a survey of the literature indicates two important things: i) whereas numerous chemicals have been shown to be hepatocarcinogenic in mice and/or rats, very few have been shown to be so in man; and ii) although the liver may be the target organ for the carcinogenicity of a particular compound in, say, the mouse, another site (eg. the urinary bladder) may be the target in another species (e.g.man). In developing an approach to establishing whether a new chemical is a carcinogen for the liver, the system of studies advocated is necessarily relevant to investigating whether the agent is a carcinogen for other sites as well.

In listing causes of liver cancer in man, Hepatitis B virus infection, leading to chronic hepatitis and cirrhosis,is the most important contributory factor (cf.chapter F). There are similarities between man and laboratory animals with respect to hepatocarcinogenesis by ionising radiation, certain sex hormones, vinyl chloride , and possibly aflatoxin B_1, but not by most other chemicals that have been shown to be liver carcinogens in laboratory animals. Consideration must be given to whether interspecies differences in the spectrum of factors that can predispose to hepatocarcinogenesis are so great that animal experiments are unhelpful in assessing whether a hazard to humans exists, or whether the apparent species differences are explained solely by the insensitivity of epidemiological methods under conditions of low levels of human exposure.

Today it is widely held that agents which facilitate the development of cancer fall into two broad types : those that act by a genotoxic mechanism and those

that act by a non-genotoxic mechanism (the term non-genotoxic is preferred to epigenetic) . This distinction is seen as fundamentally important in virtually all the sections of this report. Although, in general, more concern is attached to genotoxic than to non-genotoxic mechanisms, the potential importance of the latter type of activity for man should certainly not be lightly dismissed. The lower level of concern in the case of a non-genotoxic mechanism might be justified by a discontinuous dose-response relationship and/or by the fact that positive effects have been achieved only in animals exposed for prolonged periods to high, and often toxic, doses.

This monograph was prepared on the understanding that :

- No single laboratory test and no group of tests could definitely predict either hepatocarcinogenicity or non-hepatocarcinogenicity for man.
- The most that could be achieved would be to recommend procedures that would provide information pertinent to an understanding of the mechanism involved. Such an approach would be relevant to a more rational assessment of the hazard to man.

Chapters B to E constitute "state of the art" reviews of various aspects of hepatocarcinogenesis and in chapters F and G, based on these reviews, an attempt is made to answer the questions posed above.

The Task Force acknowledges the valuable contributions of Professor R. Wright (Southampton General Hospital, UK) and Dr. V.M. Craddock (Medical Research Council, UK) to the discussion of the aetiology of hepatic neoplasia in man, and the pathogenesis of hepatic neoplasia in laboratory rodents, respectively.

B. BIOTRANSFORMATION AND ITS RELATIONSHIP TO HEPATOCARCINOGENESIS

1. Introduction

Among the most important of the numerous physiological functions of the liver are its conversion of pro-nutrients to nutrients, and of potential toxins into harmless products for excretion in the bile or in the urine. Both functions involve a wide range of hepatocellular enzymes, some normally present in large amounts. These enzymes, and others normally present in small amounts, can be induced by a wide variety of chemicals. Many so-called carcinogens are not directly active but first require metabolic conversion (activation) into so-called proximate carcinogens with the ability to bind covalently to macromolecules, including cellular DNA. Thus, the liver cells are the ones first exposed to electrophilic metabolites which may or may not spill over to other tissues.

Enzyme induction, either by procarcinogens or by non-carcinogens, may be relevant to tumour hazard in the liver or in other organs. Thus the induction of metabolising enzymes by one agent may enhance the metabolic activation of the same, or another, agent to an electrophile. Conversely, enzyme induction may facilitate detoxification of carcinogens by a route that does not result in the production of electrophiles. There is, therefore, no simple link between enzyme induction and cancer hazard.

It is currently widely accepted that at least two kinds of mechanism are involved in carcinogenic processes generally and in hepatocarcinogenesis in particular. The first is genotoxic and involves a change in DNA itself, either at a gene or chromosome level. The second involves a wide spectrum of non-genotoxic mechanisms. (cf. J.). Methods for identifying potentially-genotoxic agents are relatively well developed, but those for defining and detecting agents acting by non-genotoxic mechanisms are poorly developed.

2. Biotransformation

Knowledge of biotransformation is fundamental for understanding both hepatotoxicity and hepatocarcinogenicity. Recently, attempts have been made to explain species differences in tumour susceptibility on the basis of metabolic data (Reitz et al.,1978; Dietz et al.,1981). In this chapter the primary aim is to highlight known differences in drug-metabolizing enzymes between laboratory animals and man, and to discuss the significance of these differences in the extrapolation of animal data to man.

As outlined above, some substances which are unreactive as such can be transformed into reactive, and ultimately carcinogenic, metabolites by mammalian enzymes. Hence, species differences in the activities of enzymes which control the concentration of reactive metabolites can lead to differences in the toxic response, and the significance of this fact for the initiation of liver tumours has been discussed by Remmer (1978). Examples of reactive metabolites (Remmer, 1978) are epoxides, radicals, quinones, and carbonium ions, among others. The functional heterogeneity of enzymes responsible for the formation and degradation of such reactive metabolites, e.g. monooxygenase (Lu,1979), epoxide hydrolase (Oesch,1978), and glutathione-S-transferase (Jakoby, 1978) is a matter of continuing research and discussion.

2.1. Age dependence

The importance of age in relation to biotransformation processes involved in the toxicity of xenobiotics has been stressed (Uehleke, 1975), and perinatal (Uehleke et al.,1972) as well as senescent (Birnbaum and Baird, 1979) changes in the drug-metabolizing enzymes of experimental animals have been well documented.

Studies on drug-metabolising enzymes in the human foetus (Rane, 1973 ; Pelkonen, 1973 ; Pelkonen et al.,1973) have shown that in contrast to the livers of laboratory animal species, the livers of aborted human foetuses contain a significant amount of mono-oxygenase (20-40% of the adult level) with no similar quantity of conjugating enzymes. This could be sufficient for the formation of ultimately carcinogenic, reactive metabolites in human foetuses (Remmer,1978). For this reason a risk of induction of hepatocellular carcinoma in the human foetus might not be adequately predicted by animal experiments (Cain,1978).

2.2. Species differences in biotransforming enzymes

The multiplicity of hepatic drug-metabolizing enzymes (Lu, 1979) complicates the problem of species differences because, in general, such differences must be expected in the qualitative composition of the isoenzymes and in the quantities of all the individual isoenzymes involved. Hence, extrapolations from one species to another regarding the metabolism and the steady-state of reactive metabolites of a particular chemical are extremely difficult.

2.2.1. Cytochrome P-450

Multiple forms of hepatic microsomal cytochrome P-450 exist, consisting of different proteins. Different forms of cytochrome P-450 are found in different animals (Guengerich, 1979). Modification of these multiple forms by preferential induction or inhibition can lead to both an increase and decrease in toxicity (Guengerich,1979). That an increase in bioactivating enzymes can result in enhanced carcinogenicity of a metabolically-activated carcinogen is plausible, but classical studies (Miller et al.,1958) have also shown the reverse in that treatment of rats with certain polycyclic aromatic hydrocarbons protected them against the hepatocarcinogenic effects of 2-acetylaminofluorene and aminoazo-dyes. These findings might reflect increased rates of detoxification.

Species differences in hepatic microsomal monoxygenase activities have been reviewed (Walker,1978). In general, small mammals exhibit higher hepatic microsomal monooxygenase activities than do larger mammals. This has been confirmed recently when metabolic clearance rates, based on body weights, of the hepatocarcinogen vinyl chloride were determined in various species including mouse, rat, the mongolian gerbil, the rhesus monkey, and man (Buchter et al.,1980). Of the species studied, man showed the slowest metabolic elimination of vinyl chloride .The importance of such species differences in the toxicity evaluation of chemicals requiring bioactivation by microsomal monooxygenases has been stressed, and a decreasing order of generation of reactive, oxidative metabolites has been postulated for some chlorinated hydrocarbons as follows : mouse>rat>man (Reitz et al.,1978; Dietz et al.,1981).

2.2.2. Epoxide hydrolase

Epoxide hydrolase (also called epoxide hydratase or epoxide hydrase) transforms epoxides into trans-dihydrodiols. Ultimately, carcinogenic epoxides are thus "detoxified", but in some circumstances, as in the formation of "Bay-region diol-epoxides" of polycyclic aromatic hydrocarbons, the enzymes also play a role in bioactivation (Oesch, 1978; Oesch, 1980-a; Lu and Miwa, 1980). With the majority of industrial aromatic and olefinic compounds it can be assumed that epoxide hydrolase simply plays an inactivating role (Oesch,1980-b). Also, because man has a much higher hepatic epoxide-hydrolase activity than many of the investigated strains of mice and rats (Oesch et al.,1974;

Oesch,1980-b), these laboratory animals could be more susceptible to toxic effects mediated by many metabolically-produced epoxides.

Epoxide-hydrolase activity is found in the microsomal and, to a lesser extent, in the cytosolic cell fractions of the liver. These two sites of activity have been shown to involve two different enzymes with different substrate specificities, and to be immunologically distinguishable from each other both in rats and mice (Guenthner et al.,1981). There is considerable individual variability in the epoxide hydrolase contents of human liver. Van Bladeren (1981) showed differences of about one order of magnitude when human liver biopsy specimens were analyzed for epoxide hydrolase.

In general, the higher epoxide hydrolase activities in man compared with those in commonly-used laboratory animal species (mouse, rat) would argue in favour of a lower sensitivity of humans for at least some hepatocarcinogenic compounds.

2.2.3. Glutathione-S-transferase

Glutathione-S-transferases catalyze the reaction of a wide spectrum of compounds with glutathione (Jakoby,1978). Although they are mostly considered "detoxifying" enzymes, certain substrates (e.g., 1,2-dihaloethanes) are converted into more toxic metabolites (Van Bladeren, 1981).

The bulk of glutathione-S-transferase activities is confined to the cytosol compartment in the liver (Friedberg et al.,1979). Liver tissues of rats and humans show very similar activities of this enzyme, e.g. when the test substrate is 2,4-dinitrochlorobenzene (Bolt et al.,1981). However, in rat liver significant activities are also associated with microsomal membranes (Friedberg et al.,1979). This localization may be of importance for inactivation of very lipophilic epoxides which are concentrated within the lipid membranes. In the human liver, membrane-bound microsomal glutathione-S-transferase activity, in contrast to cytosolic activity, is only about 1/10 of that found in the rat (Bolt et al.,1981).

It has been suggested that the epoxide formed from vinyl chloride in the endoplasmic reticulum is partially detoxified by the membrane-bound glutathione-S-transferase. The presumed urinary metabolite resulting from this pathway is hydroxyethylmercapturic acid, which

represents a much smaller fraction of the urinary vinyl chloride excretion products in humans exposed to vinyl chloride than is the case in exposed rats (Müller et al.,1980). Consequently, low levels of the epoxide of vinyl chloride may saturate the capacity of this deactivation pathway in the human liver, but not in rat liver (Bolt et al.,1981).

2.2.4. Conclusions

From the above selected points it appears that a general procedure cannot be proposed for extrapolation of quantitative hepatocarcinogenicity animal data to man. A reliable procedure can be worked out only on a compound-by-compound basis. A prerequisite is the detailed knowledge of "activating" and "inactivating" pathways of biotransformation for the compound in question, and of the enzymes involved. Although for a series of hepatocarcinogenic compounds man seems to be less susceptible than many experimental rodents, the reverse might be true for other chemicals(Popper,1978). Great efforts are being made in this area of research, but the complexity of the metabolic problems involved makes progress toward practical solutions slow.

2.3. The role of metabolic overload

2.3.1. Dose dependent pharmacokinetics

In current test protocols for long-term animal bioassays the administration of large doses of the test chemical is sometimes recommended, the intention being to counteract the poor sensitivity of the test method and allow a statistical evaluation of the tumour incidences found in the treated and control animals. The administration of lower doses would necessitate the use of a very large number of animals. Another assumption is that the carcinogenic effect of a chemical is dose-dependent. A high dose should increase the incidence of tumours and shorten the latency period. However, the pharmacokinetics of the compound should not be neglected in the interpretation of the dose response in long-term animal bioassays (Dietz et al.,1981; Gehring et al.,1976; Gehring et al.,1978).

It is accepted that many metabolic pathways are saturable so that "linear" pharmacokinetics can be applied only in a defined concentration range. Recent investigations into the dose-dependence of the metabolic elimination of volatile, inhaled xenobiotics now allow the

distinction of groups of compounds exhibiting different behaviour patterns, (Filser and Bolt, 1981) as follows :

a) Strict Michaelis-Menten kinetics apply to the metabolic elimination of dioxane (Young et al.,1978), methyl vinyl ether (Andersen et al.,1980) and acetone (Filser and Bolt, 1981) which are relatively polar solvents.

b) A second group of chemicals exhibits saturation characteristics at higher concentrations, as in a Michaelis-Menten function, but shows a first-order pattern (probably perfusion-limited) in lower concentration ranges. Members of this group are benzene (Andersen et al.,1980) and halogenated ethanes and ethylenes (Filser and Bolt, 1981).

c) Compounds may also be eliminated according to a first-order rule over the entire experimental range of concentrations (e.g., methyl bromide, Andersen et al.,1980). Theoretically, saturation would occur at practically lethal concentrations.

Thus species differences in phenomena such as saturation characteristics may explain species differences in carcinogenicity. For instance, recent studies (Elcombe et al., 1982) on the dose-dependent metabolism of trichloroethylene in rats and mice have shown that saturation of metabolism occurs in rats but not mice (i.e., rats belong to group b, above, and mice to group c).

The Food Safety Council (USA, 1978) has recommended that metabolic and kinetic data should be taken into account when choosing the doses for a long-term bioassay. For compounds that are carcinogenic by metabolic conversion into reactive metabolites, the following cases should be considered :

a) Saturation of an activating pathway. In this case a dose higher than that resulting in a maximal effect will not increase the rate of transformation to toxic metabolites, and hence the tumour rate. As an example, the induction of hepatic angiosarcoma in rats exposed to vinyl chloride was found to be entirely related to the amount of carcinogen biotransformed, and not to the concentration applied (Gehring et al.,1978).

b) Overwhelming of a de-activating pathway . This would mean that the tumour risk will increase disproportionally with increasing doses above the point of metabolic saturation.

c) Alternative pathways which could show dissimilar saturation characteristics. An indication of this phenomenon is a variation in the ratio of various excreted metabolites as a function of the applied dose. The effect on the resulting tumour risk would then depend on the activation and inactivation characteristics of the different competitive pathways.

2.3.2. Repair mechanisms

Electrophilic metabolites covalently bound to the genetic material could lead to tumour initiation when the lesion is not efficiently repaired. There has been speculation (Gehring,1977) that the repair system follows Michaelis-Menten kinetics. On this basis a disproportionate increase in primary DNA-lesions with higher doses of a carcinogen is feasible.

Recently it has been demonstrated that different cell populations of rat liver show different DNA repair activities (Lewis and Swenberg,1980). This is also true for hepatic, and several extrahepatic, tissues (Kleihues et al.,1978). Thus, there may be differences in susceptibility to carcinogens between tissues, or even between different cells within tissues, when widely different doses are administered.

The establishment of repair kinetics is a very time-consuming task at present and more data in this field will become available when new techniques for the immunochemical detection of DNA lesions have been developed.

C. THE ROLE OF FACTORS DISTINCT FROM THE TEST CHEMICAL IN THE PATHOGENESIS OF LIVER NEOPLASIA IN LABORATORY RODENTS

1. Introduction

A necessary background to any consideration of hepatocarcinogenicity is a knowledge of the background liver tumour incidence in rats and mice, and recognition of the fact that a number of non-specific factors have been found to influence this incidence.

The subject has been made more difficult because definitions have been changing and, even today, pathologists differ widely in the terms they use and the criteria they rely on for distinguishing between neoplastic and non-neoplastic lesions and between benign and malignant lesions. At one extreme there are pathologists who are prepared to apply the term "hepatocarcinoma" to groups of as few as 2 or 3 abnormal liver cells .Close to the same end of the range are pathologists who have dispensed with the terms "hyperplastic nodule" and "benign hepatoma" and regard all proliferative lesions as malignant. At the other extreme are pathologists who require to see evidence of metastasis before accepting a diagnosis of malignancy.

Between these extremes lies the majority view, amounting almost to a consensus, as follows :

i) expansive lesions exhibiting evidence of proliferative activity, invasiveness and/or metastasis, and certain architectural and cytological features, should be regarded as malignant neoplasms;

ii) expansive lesions larger than an individual lobule, exhibiting only proliferative activity without cytological evidence of dedifferentiation, and an absence of invasiveness and/or metastasis, should be regarded as benign neoplasms. Benign liver neoplasia as a response in rodents should not be regarded as insignificant. Various workers have defined their criteria for distinguishing between benign and malignant neoplasia (Walker et al.,1973; Squire et al., 1975);

iii) hyperplastic nodules may in theory be present but they are impossible to distinguish unequivocally from benign neoplasms unless evidence of a repair phase following previous damage, e.g.fibrosis, is present.

In response to exogenous agents the livers of rats and mice may develop localised areas of altered hepatocytes, and/or altered cellular arrangement, and the cells in these areas may exhibit alterations in certain enzyme activities. Enzyme-altered islands can be detected by histochemical methods in livers which appear more or less normal in haematoxylin and eosin-stained sections. In some cases, at least, these islands appear to be clones of cells

and it has been suggested that they differ from normal hepatocytes because a stem-cell from which they originated has mutated on exposure to a genotoxin. Alternatively, they may represent no more than collections of cells that have suffered similar damage or manifestations of adaptation. When, during embryogenesis, a pluripotent stem-cell differentiates into a more specialised cell, structural and functional changes occur such that the differentiated cell appears to be different from the progenitor cell. The significance of enzyme-altered foci is discussed in more detail in chapter D.3. Cairns (1981) has recently discussed non-genotoxic mechanisms in relation to cancer and his paper appears to be relevant to any in-depth consideration of the pathogenesis of enzyme-altered islands and other liver changes.

Another source of confusion in hepatocarcinogenicity is that with the increasing availability of SPF animals, more untreated controls are living long enough to develop tumours. Also, the quality of basic diets has been changing in the direction of higher fat concentrations to counteract the tendency of diets to crumble when pasteurized. At the same time animal breeders have been selecting for rapid growth and large litter size. In doing so they may also have, inadvertently, been selecting for propensity to develop tumours spontaneously. In any event, obesity has become widespread among laboratory rats and mice and associated with this there is an "epidemic" of neoplasms of all kinds, including liver tumours in mice (Roe,1981).

2. Spontaneous Liver Tumour Incidence

2.1. In rats

The impression given in IARC's "Pathology of tumours in laboratory animals" (IARC, 1976) is that spontaneous liver tumours are rare in rats. However, it is clear that the IARC report is out of date since the references quoted range from 1909 to 1940. Also, there is no recognition of the accumulating evidence of widely-different incidences encountered in rats of different strains.The current situation is that, depending on the strain and the conditions, liver tumours are not particularly uncommon in elderly, untreated SPF rats fed ad libitum. This is evinced by several recent reports (Table 1).

TABLE 1

Percentage Incidences of Liver Tumours of Untreated Two-year-old Rats of Different Strains.

Strain	Sex	Number examined	% with hyperplastic nodule*	% with liver neoplasia	Reference
Wistar (Colworth strain)	M	45	13	16	Roe, unpublished data
	F	45	11	29	
Sprague-Dawley	M	85	7	2	Kociba et al.,1978
	F	86	9	1	
Sprague-Dawley	M	86	0	4	Kociba et al.,1979
	F	86	5	2	
Fischer 344	M	1794	1.3	0.4	Goodman et al.,1979
	F	1794	2.7	0.4	

*Benign and/or malignant.

These figures very clearly indicate that the occurrence of liver tumours in carcinogenicity studies in rats is not necessarily confined to treated groups.

2.2. In mice

Liver tumour incidences of up to 100% may occur in some strains such as C3H. It is not uncommon for tumour cells to exhibit prominent eosinophilic cytoplasmic inclusions consisting of whorls of smooth endoplasmic reticulum, the significance of which is unclear.There is no indication that they are viral in nature. The enzymic profiles of the lesions exhibiting eosinophilic, cytoplasmic inclusions has yet to be determined. In mice generally, spontaneous tumours are much more common in males than in females. In strains where they occur in up to 100% of animals they appear earlier in males than in females. Finally, in response to many agents which enhance the incidence of liver tumours in mice, the effect is much more evident in males than in females.

3. Non-Specific Factors Known to Influence Liver Tumour Incidence in Rats and Mice.

Various exogenous and endogenous factors are considered to play a major role in tumour incidence. These factors include the quality and quantity of the diet, the endocrine and microbiological status, and the influence of stress and genetic differences.

3.1. Quality and quantity of the diet

3.1.1. Diet restriction versus ad libitum feeding

Recent papers by Tucker (1979) and Conybeare (1980) indicate that in mice fed on standard laboratory diets, reductions in liver tumour incidence of up to 8-fold may occur in response to diet restriction of the order of 10-25%. The effect is apparent for malignant as well as benign liver cell tumours, and similar beneficial effects on the incidence of a wide variety of other tumours, both benign and malignant, are seen. Life-time expectation of tumour development decreases despite increased longevity.

Tucker (1979) reported marked reductions in tumour incidence in response to mild dietary restriction in rats, but the sites affected were mainly the pituitary and mammary gland, although the incidence of tumours at many other sites were also reduced. In the strain of rats used, spontaneous liver tumour incidence was negligible even in ad libitum fed animals, so that there was no scope for reduction of their incidence by dietary restriction. No study could be found concerning the effects of dietary restriction on liver tumour incidence in a strain of rats with a high spontaneous incidence of such neoplasms under ad libitum feeding conditions.

Ross (1969) studied the effects of age and diet restriction on hepatic enzyme patterns in the rat. He found that with long-term calorie restriction the levels of the biochemical indicators, and the cell and animal size, were more like those of the young rat, and that the longest life spans were obtained.

Any factor, e.g. diet restriction, which influences liver enzyme profile may alter the response of rodents, in either direction, to the spontaneous development of liver tumours, and may also alter the response and susceptibility to exogenous hepatoxins and potential carcinogens. Specific dietary elements such as vitamins have been shown to influence the tumour response to carcinogenic compounds in

other tissues. The possibility that a similar phenomenon applies to the liver may have to be considered. There is an urgent need for further research in this area to elucidate possible mechanisms whereby these factors exert this effect.

3.1.2. Dietary fat content. Gellatly (1975) showed that by increasing the fat content of a semi-synthetic diet, liver tumour incidence in inbred C57BL mice could increase substantially.

3.2. Endocrine status and stress

The liver is a target organ for tumourigenicity in response to various natural and synthetic sex hormones in mice, rats and other species. Several observations suggest that the effects of dietary restriction on the incidence of various tumours in rats and mice is not directly attributable to calorie restriction, but to an effect on endocrine status that is possibly secondary to the stress which animals experience on being faced with an empty food basket (Roe,1981).

3.2.1. Influence of hormonal status on tumour incidence

a) Spontaneous tumours. Generally, male mice have a greater spontaneous incidence of hepatocellular tumours than do females. This sex difference is also evident in other species, but is not usually so clear-cut (Toh,1973). Hypophysectomy in C3H/YBR F1 mice reduces the incidence of tumours, normally about 100%, to zero (Heston,1963) but this may be due to a decrease in food intake. Sex hormone manipulation may influence spontaneous liver-tumour incidence in both rats and mice (see also chapter E-2.3).

As suggested in chapter D.2.3.2, increases in ploidy appear to be involved in the carcinogenic process, and it is worthy of note that male animals have higher liver-DNA content and a greater degree of ploidy than do females. This sex difference appears to be due to neonatal hormone patterns (neonatal imprinting) rather than to the hormonal background in adult life (Toh,1971,a-b).

b) Chemically-induced tumours in rats and mice. Males tend to be more susceptible than females to agents that enhance the incidence of liver tumours and, in general, endocrine ablation reduces the higher male susceptibility. Ovariectomy and testosterone administration enhance liver tumour risk in females, and orchidectomy and estrogen administration reduces risk in males (Andervont, 1950; Agnew and Gardner,1952, Warwick,1971).

Orchidectomy combined with oestrogen administration suppresses the hepatocarcinogenicity of nitrosoethylurea, while hypophysectomy protects against aflatoxin B_1 and acetylaminofluorene hepatocarcinogenicity (Warwick,1971). Since feminisation generally protects against tumourigenicity, it is frequently assumed that alterations in xenobiotic-metabolising enzymes (e.g, cytochrome P-450) are responsible for changes in carcinogenic response because most mixed-function oxidase activities are higher in male rats.
Evidence exists, however,that hormones may influence tumour incidence by effects on tumour progression or expression rather than by increasing the likelihood of tumour initiation in certain non-hepatic tissues (Shama and Criss,1978; Furth,1975), but there are few studies of the effects of hormones on the progression of liver tumours. One such study demonstrated that castration of male mice several weeks after treatment with urethane reduced the normally-observed sex difference in carcinogenicity (Vesselinovitch and Mihailovich, 1967).

3.2.2. Stress and tumour incidence

Stress of various types may significantly affect the incidence of both spontaneous and chemically-induced tumours.Generally, stress (e.g.that associated with unsuitable housing, temperature, noise, starvation) affects some or all of the following: adrenocortical activity, gonadal hormone secretions and neurotransmitters. Conflicting data exist, but most studies indicate that increases in adrenocortical activity occur (Brain and Benton,1979; Riley,1981), which in turn lead to effects upon other endocrine systems. Stressful conditions have been shown to increase plasma concentrations of corticosteroids, resulting in the induction of hepatic enzymes such as tyrosine aminotransferase (Omrani et al.,1980).

Stress may affect tumour incidence by altering the initiation or promotion/progression stage of tumour development. The initiation stage often depends upon the balance between detoxification and toxification of chemicals (cf.chapter B). Several studies have shown alterations in mixed-function oxidase activity under conditions of stress (Kato,1977). It is apparent that such effects of stress depend on an intact pituitary/adrenal axis. Significantly, rat hepatic aryl-hydrocarbon hydroxylase and epoxide hydrolase increase as a result of overcrowding stress (Capel et al.,1980). This study also showed a decrease in the microsome-catalysed binding of benzo(a)pyrene

to DNA. Stress by intraperitoneal injection of Celite^{R} caused an increase in N-oxidation at the expense of N-demethylation (Arrhenius,1968). It is noteworthy that corticosteroids raise the incidence of 2-aminofluorene-induced tumours (Weisburger and Weisburger,1963), presumably by increasing the metabolic activation of 2-aminofluorene, while adrenalectomy lowers the liver tumour incidence.

Several studies suggest the involvement of corticosteroids, directly or indirectly, in the progression of tumours. Adrenalectomy leads to an increase in DNA synthesis, and in mitosis and ploidy of rat liver hepatocytes, while, conversely, corticosterone leads to a decrease in these parameters (Desser-Wiest,1974). Thus corticosterone appears to have a negative regulatory effect upon nuclear and cellular division in the rat liver. This regulatory effect of corticosterone can be overcome by progesterone, which is thought to compete with corticosterone for its hepatic receptor (Desser-Wiest,1979). The life-span of rats treated with diethylnitrosamine is shortened by the simultaneous administration of progesterone . Deaths were related to liver tumours. The author suggests that progesterone acts as a co-carcinogen by inhibiting endogenous corticosteroid activity thereby enhancing mitosis and cell proliferation.

3.3. Microbiological status

The age-standardized incidence of spontaneous liver tumour development in C3H was lower in germ-free animals than in conventionally-maintained animals (Roe and Grant,1970). This may have been the result of differences in nutritional or endocrine status. The fact that germ-free status more or less abolished the liver-tumour enhancing effect of 7,12-dimethylbenz(a)anthracene merits further study.

3.4. Partial hepatectomy

Partial hepatectomy has long been known to enhance liver tumour incidence in rats exposed to known hepatocarcinogens (Craddock,1977; Tatematsu et al.,1977), but there is no evidence that it enhances liver cancer risk in the absence of concomitant exposure to a hepatocarcinogen. It is widely assumed that the same would be true in other species but little experimental work has been reported.

D. MECHANISMS OF CARCINOGENICITY

The currently accepted theories of chemical carcinogenesis imply a sequence of events of both genetic and non-genetic nature. Genetic mechanisms involve the formation of a primary lesion at the DNA level by an electrophile, followed by either repair of that lesion or fixation by cell replication which converts the alkylated site into a mutated site. Later, this may be followed by one or more of a wide variety of "non-genetic" processes which involve further cell divisions but are generally poorly understood at the molecular level. It is appropriate to consider separately the "genotoxic" and "non-genotoxic" mechanisms relevant to the generation of liver cancer.

1. Genotoxic Mechanisms

For the relevant details of this matter, references to the extensive original literature can be obtained by consulting recent reviews (Sarma et al.,1975; Craddock,1976; Lawley,1977; Pegg,1977; Rossman et al, 1977; Roberts,1978; Grover,1979; Grunberger and Weinstein, 1979; Singer and Kröger,1979; Seeberg,1981).

Only the most relevant points are summarised here.

1.1. Binding to DNA

Electrophilic, ultimate carcinogens react with nucleophilic sites in DNA, chiefly at nitrogen and oxygen atoms in the purine and pyrimidine bases and at the oxygen atom in the phosphate moiety, to form a wide variety of adducts. The comparison of products formed by various carcinogens in target and non-target tissues has provided clues as to which may be the most relevant reaction products. Obviously there are biological differences between the alkylation of DNA by "bulky" molecules (e.g., reactive metabolites of aflatoxin B_1, acetylaminofluorene or polycyclic hydrocarbons) and by the smaller methyl or ethyl groups. In general, the former cause frame-shift mutations in bacterial test systems whereas the latter result in base-pair substitutions.

For methylating and ethylating carcinogens the critical target is believed to be the O-6 of deoxyguanosine, although many of these agents form high proportions of 7-alkylguanine in DNA, depending on the chemical nature of the reactive metabolite and the reaction mechanisms involved. However, the correlation between the amount of relevant

product formed and the initiation of cancer is not perfect. For example, one injection of dimethylnitrosamine (DMN) into a rat does not induce liver cell cancer, although a relatively large amount of the putative relevant product, O-6-methylguanine, is formed. But if the single injection of DMN is given at the time that restorative hyperplasia is taking place after partial hepatectomy, then one injection will induce cancer. This, and much other work, strongly suggests that the extent of cell replication occurring at the time of exposure to the carcinogen is important.

The pattern by which methylating and ethylating carcinogens react with DNA contrasts to that of more "bulky" compounds.For instance, the reactive diol-epoxide of benzo(a)pyrene reacts preferentially with the exocyclic amino-groups of the nucleobases (N-2 of guanine, N-6 of adenine, N-4 of cytosine) and leads to the formation of a variety of isomeric alkylation products. N-acetoxy-acetylaminofluorene binds preferentially to N-2 and C-8 of guanine, whilst the epoxide formed metabolically from aflatoxin B_1 is attached to N-7 of guanine.For several N-7 alkylation products of deoxyguanosine in DNA (products of methylation and ethylation, as well as the aflatoxin B_1 reaction product) it has been proposed that alkylation of this particular position results in labilisation of the N-glycosidic bond of the deoxyguanosine within the DNA molecule. This would produce an apurinic site and, in consequence, would eventually lead to miscoding when cell replication occurs. Apart from this possibility it is not clear whether any alkylation of DNA by "bulky" molecules could lead to direct consequences of miscoding at the next cell replication.

By contrast, there are sufficient data (combination of polynucleotides in vitro; coding patterns of modified polynucleotides in transcription or translation processes) demonstrating that methylation or ethylation of bases at or near the "Watson-Crick" sites of hydrogen bonds results in significant miscoding events. This may explain the high incidence of base-pair substitutions caused by these agents in bacterial test systems and provide the molecular basis for the "genotoxic" mechanism of methylating and ethylating carcinogens.

A general consequence of a massive alkylation of DNA is the inhibition of replication and transcription, such inhibition constituting the mechanism of action of alkylating cytostatics. Where direct mispairing occurs, there is also an effect of DNA alkylation on the action of

DNA-polymerases : DNA-synthesis is much slower than normal, but the newly-synthesised DNA contains the coding errors.

1.2. DNA repair

There is strong evidence that when alkylation by small molecules occurs, the replication of damaged DNA is the crucial event in initiating cancer. This means that the time during which the DNA remains damaged in the cell is important. If the cell repairs the damage before replication occurs there is no permanent harm. On the other hand, if replication occurs before the damage has been repaired, the alteration in DNA may be "fixed" in the daughter strands and a permanent inheritable change, i.e. a mutation, may have taken place.

When such a genotoxic chemical carcinogen is absorbed and metabolised in the appropriate way, the later development of neoplasms depends inter alia on at least three factors :

a) the nature and extent of DNA damage;
b) the rate of cell replication, or more specifically of DNA replication, in the target cell;
c) the rate and fidelity of repair of DNA damage.

When the repair rate is high, as in the liver, one treatment with alkylating agents does not normally induce cancer unless the rate of cell replication is increased as, for example, by partial hepatectomy. Where the ability to repair DNA damage is low, as in the brain of adult rats, one treatment does not induce tumours because the cells in the adult brain are not replicating. However, the neonatal brain in which the nerve cells are dividing is possibly the most sensitive organ in the animal for induction of cancer by compounds such as nitrosomethylurea. The kidney occupies an intermediate position in this respect when compared with liver and adult brain. Repair is slower than in the liver, but replication in the target kidney cells is faster. One treatment with a carcinogenic alkylating agent can induce a low incidence of kidney tumours.

Most of what has been considered above refers to direct methylating or ethylating agents, but the underlying principles may also hold true for other chemical carcinogens, i.e. when replication of DNA damaged by a chemical leads to a permanent change in the daughter strand. A repairable lesion is converted at replication into a non-repairable,

inheritable change in base sequence. This "fixation" of damage at replication may occur by a variety of mechanisms :

a) Mispairing of bases, as discussed above (1.1.) Thus, guanine pairs with cytosine, and O-6-methylguanine pairs more readily with thymine, so that replication of DNA containing O-6-methylguanine can cause a G-C to A-T transition.
b) Aflatoxin and 4-acetylaminofluorene form DNA adducts which are excised, leaving apurinic sites in DNA. It has recently been shown that such sites may be mutagenic and therefore possibly carcinogenic.
c) Carcinogens may induce error-prone repair in which the so-called repair process itself produces an altered DNA. This may be due, as in bacteria, to inhibition of a "proof reading" enzyme.
d) Carcinogens may induce errors in post-replication repair, a process analogous to recombination in bacteria. Although often postulated as a mechanism of action of a series of "bulky" carcinogens, e.g. polycyclic hydrocarbons, there is no direct evidence for this.

Finally, it is possible for chemicals to cause a change in DNA at replication by reacting, not with the DNA itself, but with the machinery for its replication, especially with the DNA polymerases. Normally these enzymes make "mistakes" in base-pairing at a rate of only 1 in 30,000. Metal compounds, especially those which are carcinogenic, have been shown to enhance this error frequency (Seeberg, 1981).

2. Non-Genotoxic Mechanisms

2.1. Liver injury involving necrosis

The role of hepatocellular injury in determining the development of hepatic neoplasia attracted attention probably because of the association between cirrhosis and the development of hepatocellular carcinoma in man (MacSween and Scott,1973). In an early study by Edwards and Dalton (1942) it was shown that repeated administration of carbon tetrachloride (CCl_4) to mice produced extensive centrilobular necrosis and led eventually to chronic lesions where the regenerated parenchyma was divided by connective tissue strands into discrete nodules which they called "hepatomas". These lesions were transplantable in mice of the same strain and were regarded by the authors as neoplasms. Although a high incidence of transplantable lesions of this sort occurred in mice

when injections of CCl_4 were maintained for two months or longer, no such lesions were observed when the same treatment lasted less than two months, indicating a clear threshold of response (Edwards and Dalton,1942). It was found that when three large doses of CCl_4 were given within two to three weeks, no hepatomas were produced even though extensive hepatocellular necrosis was evident. When the same total dose was divided into a large number of smaller hepatotoxic doses, administered 2-3 times weekly for approximately two months, a high incidence of hepatomas occurred, indicating the importance of prolonged insult in the induction of these tumours. Such phenomena also occur in rats. CCl_4 administered subcutaneously in high doses produces extensive cirrhosis in the rat. Early neoplastic lesions were observed in the cirrhotic animals (Reuber and Glover,1968). CCl_4 is rapidly metabolised by the microsomal enzyme system of the rat liver to active intermediates that bind covalently to hepatic macromolecules. Only a low level of DNA binding was found even when a large dose of CCl_4 was given (Diaz Gomez and Castro,1980-b) and there are claims that no DNA binding occurs at all (cf. Appendix 2). Furthermore, CCl_4 has not been shown to be mutagenic (Simmon et al.,1977) and there is some evidence that it does not interfere with the process of DNA repair (Rickart et al.,1980).

Eschenbrenner and Miller (1945) further investigated the role of chronic injury in the production of hepatocellular tumours. They employed a series of ascending doses of chloroform ($CHCl_3$) and found that hepatomas developed only in animals treated with doses high enough to produce hepatocellular necrosis: all the animals that developed hepatomas also had cirrhosis. The authors concluded that the "cycle of necrosis and regeneration produced by each successive dose was responsible for the production of hepatomas".

It has been confirmed (Reitz et al., 1980) that $CHCl_3$ produces liver tumours when administered at dose levels which lead to severe tissue damage in short-term studies, whereas tumours do not develop when the doses administered produce no tissue injury. The same authors demonstrated that $CHCl_3$ neither combines covalently with DNA, nor induces DNA repair. Like CCl_4, $CHCl_3$ is not mutagenic to bacteria when tested either in the liquid or vapour form (Van Abbe et al.,1982). These experiments indicate clearly that repeated episodes of hepatocellular necrosis and regenerative hyperplasia are liable to result in neoplasia in this organ, despite the fact that the compounds responsible are non-mutagenic.

There are clear examples in other tissues where a similar pathological process involving repeated cell injury and repair leads to the development of cancer. Thus persistent injury to the subcutaneous tissue of rats and mice, if maintained for several months, may result in the development of local sarcomas (Grasso and Golberg,1966), while the surgical implantation of chemically-inert foreign bodies in the urinary bladder of rats and mice induces transitional cell carcinoma if they are left in situ for several months (Bryan and Springberg,1966; Weil et al,1967;Ball et al.,1964; Roe,1964). In both these instances, cell proliferation (i.e. fibroblasts in subcutaneous tissues and urothelial cells in the bladder) is maintained for prolonged periods. It seems reasonable to suggest that hepatic tumours which develop after a series of episodes of necrosis and regenerative hyperplasia may have no more significance in terms of possible human cancer hazard than in the two examples given above.

A somewhat similar line of reasoning was adopted by the FDA in evaluating the induction of liver cancer in rats by selenium. At very high doses, this element produces extensive hepatocellular injury and cirrhosis. However, it was not considered to present a carcinogenic hazard to man mainly because chronic tissue damage was thought to have played an important role in the pathogenesis of the cancers (FDA, 1974).

2.2. Liver injury at subcellular level.

Liver damage may not necessarily manifest itself as liver cell necrosis. de Duve and Wattiaux (1966), Kerr (1967), and Arstila and Trump (1968) have indicated that damage to this organ may occur at subcellular level, while Parke and Gray (1978) showed that there may be a biochemical malfunction involving certain enzymes.

The hypothesis that continued damage to the liver at subcellular levels may lead to multiple areas of hyperplasia, and eventually to carcinomas, stimulated other investigations. Ponceau MX and Safrole, both of which induced various subcellular and biochemical changes indicative of hepatocellular damage in the rat (Crampton et al.,1977-b) were shown to induce hepatocellular tumours in long-term tests. In sequential studies both compounds initially appeared to induce an "adaptive" change in the liver that was lost after a few weeks of continued administration.This was followed by biochemical and histochemical evidence of hepatotoxicity. With continued administration, hepatotoxicity became progressively more pronounced and was followed soon after by the

appearance of multiple nodular areas that corresponded morphologically to the hyperplastic nodules (Crampton et al.,1977-b; Grasso and Gray, 1977), some of which progressed to a well-differentiated trabecular carcinoma. Such lesions metastasised to the lung (Grasso and Gray,1977).

In a dose-response experiment carried out on Ponceau MX, a high incidence of tumours was obtained in animals fed with hepatotoxic levels in the diet, whereas no tumours were obtained at lower levels where the hepatotoxic effect was equivocal or absent (Grasso, 1979). Phenobarbital and butylated hydroxytoluene induced mono-oxygenase activity, and both failed to induce hepatocellular nodular lesions when administered to rats at relatively high doses and for the same length of time (85 weeks) as Ponceau MX and Safrole (Crampton et al.,1977-a). Sequential studies revealed that the level of mono-oxygenase activity was high, and remained constant throughout the period of observation. There was no evidence of damage at any time during the study.

In another study, prolonged high-dose treatment of rats with phenobarbital for up to three years induced benign liver tumours (Rossi et al.,1977). In a subsequent investigation with rats maintained for the same length of time and on a similar treatment, hepatocellular necrosis and nodular lesions developed. The lesions were diagnosed as hyperplastic nodules but might be considered as neoplasms by other pathologists (Butler,1978).

The mechanism which operates when damage occurs at the subcellular level is less clear. Since Ponceau MX and Safrole were not mutagenic for micro-organisms, either with or without metabolic activation (Rowland and Grasso,1977), it seems reasonable to assume that the cell damage in intact mammals was limited to extra-nuclear components and did not directly involve nuclear DNA. In other words, the tumours that arose were probably the result of a non-genetic rather than a genetic mechanism.

An insight into a possible mechanism might be obtained by considering the DNA changes that accompany liver enlargement, since compounds which produce damage at subcellular levels appear also to produce enlargement of this organ (Crampton et al.,1977 a-b; Schulte-Hermann,1974; Butler,1978). These changes occur at a very early stage during studies with compounds exerting this effect, and tumours develop only after long periods of administration. It is not clear whether shorter-term

administration of the chemical, followed by withdrawal, results in an increased liver tumour incidence. However, chemically-induced liver changes such as hepatomegaly are readily reversible on cessation of exposure.

There is a gradual increase in the number of polyploid hepatocytes, apparently as part of the natural process of ageing in both rats (Alfert and Geschwind,1958) and mice (Schwartz,1967), but this phenomenon appears to be accelerated by compounds that produce liver enlargement (Schulte-Hermann,1974; cf. Appendix 1). The increase in the content of nuclear DNA which an increase in ploidy implies, results from reduplication of the DNA without the occurrence of mitotic division (Brodsky and Uryvaeva,1977). On this basis it appears plausible to consider that the increase in ploidy level seen in liver enlargement can be compared to the increase in mitotic activity which occurs in regenerative hyperplasia, in that both processes involve a phase of DNA replication.

These experiments suggest that chronic tissue injury at subcellular levels in the rodent liver is in some way involved in the genesis of tumours. If this is the case it could account for the carcinogenic activity of a large number of non-mutagenic chemicals such as most of the organochlorine (IARC, 1974-1979), hypolipidaemic (Cohen and Grasso,1981) and porphyria-inducing compounds (De Matteis,1978), and the phthalates (NTP, 1982). The majority of these compounds give rise to liver enlargement accompanied by ultrastructural changes (e.g. SER hypertrophy, disturbance of lysosomal pattern and peroxisome proliferation) but no overt histopathological evidence of cell injury in short-term tests. Liver tumours in life-time rodent studies have been reported for these compounds. The biochemical disturbances which they produce are at present ill-defined and their significance is even less well understood.

Despite concern over chemically-induced hepatomegaly it should be noted that a two- to three-fold liver enlargement occurs as a physiological change during pregnancy in rodents (Wilson et al.,1970)

2.3. The significance of hyperplasia

As mentioned in the preceding section, hyperplasia may play a determining role in the production of tumours (Farber,1978).There has been considerable speculation on the role played by an increase in mitotic activity as a determinant of tumour development. The most plausible hypothesis seems to be that which connects hyperplasia with the occurrence of somatic mutation. Such mutations may arise

spontaneously (Atwood and Scheinberg,1958; Drake, 1977) or as a result of exposure to environmental carcinogens. If the mutated cells have some survival advantage it would be logical to assume that their number would increase if the rate of cell multiplication were enhanced. Such cells may eventually become the source of cancer cells since it is believed that under adverse growth conditions cancer cells have some survival advantage over other cells (Vasiliev et al.,1962). The factors responsible for the increase in mitotic activity involved in cell replication after injury, or possibly the increase in ploidy in liver enlargement, may act as promotional events which considerably enhance the probability of tumour development from these cells.

2.4. The significance of peroxisome proliferation

The administration to rodents of a variety of chemicals (e.g. diethylhexyl phthalate, ethylhexanol, clofibrate, nafenopin, tibric acid and gemfibrozil) leads to hypolipidaemia, hepatomegaly, proliferation of hepatic peroxisomes, and smooth endoplasmic reticulum (Hess et al.,1965; Svoboda et al.,1967; Reddy and Krishnakantha,1975; Moody and Reddy,1978). The increase in peroxisome numbers is accompanied by increases in catalase, H_2O_2-generating oxidases, carnitine acetyltransferase and long-chain fatty acid-oxidation enzymes (Reddy and Krishnakantha,1975; Osumi and Hashimoto,1979). A novel form of hepatic microsomal cytochrome P-450 with a high specificity for the hydroxylation of lauric acid is also induced (Orton and Parker,1982). Many such agents induce hepatocellular carcinoma in rodents (Reddy and Rao,1977; Reddy et al.,1979 and 1980; Svoboda and Azarnoff,1979; NTP,1982) with, where studied, no evidence that a DNA interaction or mutagenic event has occurred (Von Dänicken et al.,1981; Warner et al.,1980).

It is noteworthy that high-fat diets elicit peroxisome proliferation and concomitant increases in peroxisomal β-oxidation (Ishii et al.,1980; Neat et al.,1980). Furthermore, high-fat diets also increase tumour incidences in rodents (see chapter C). Other agents which raise the hepatic lipid content, e.g. the hepatocarcinogen ethionine, are also peroxisome (microbody) proliferators (Steiner et al.,1964; Wood,1965). Tri-iodothyronine, which mobilizes lipid from adipose tissue and enhances the transport of lipid into the liver,also elicits peroxisome proliferation in the rat (Fringes and Reith,1981). It is possible that any chemical or process which results in increased intrahepatic lipid

leads to peroxisome proliferation due to an adaptive (homeostatic) response of the animal.

The link between peroxisomal proliferation and hepatocarcinogenesis in rodents is at present tenuous. Reddy and coworkers (1979,1980) have postulated that "unbalanced" increases in peroxisomal enzyme activities lead to an abnormally high steady-state concentration of H_2O_2, which may in turn lead to genotoxic and cytotoxic damage due to the formation of reactive oxygen species. Indeed, lipofuscin, a lipid peroxidation product, is often observed in liver cells after treatment of animals with peroxisome proliferators (Reddy et al, 1982).

An aspect of the biology of peroxisome proliferators which has received less attention is their frequent ability to raise mitotic activity and cell proliferation in liver (Reddy et al.,1980;Izumi et al.,1981). Both effects have been frequently associated with subsequent increased liver tumour incidence (see section 2.3). Furthermore, certain hypolipidaemic agents have been shown to promote diethylnitrosamine-induced hepatic carcinogenesis (Reddy and Rao,1978).The promotional effects of such agents in terms of cell proliferation may be contributory, if not essential, to the carcinogenic process. The hepatic effects of peroxisome proliferators appear to be species-specific. For example, clofibrate causes liver enlargement, peroxisome proliferation and increased lauric acid hydroxylation in rats but not in marmosets (Holloway et al.,1980; Orton and Parker,1982). Hence, if the hepatomegaly and peroxisome proliferation elicited by clofibrate and related compounds are associated with the increase in the incidence of rat hepatocellular carcinoma, it is unlikely that marmosets will develop liver tumours after treatment with these compounds. Limited evidence from studies with the hypolipidaemic agents clofibrate and gemfibrozil in humans indicates the absence of peroxisome proliferation and liver cell tumours after treatment (Hanefeld et al.,1977; de la Iglesia et al.,1981; IARC,1980). A more thorough discussion of species differences and relevance to man can be found in the review by Cohen and Grasso (1981).

3. Enzyme-Altered Foci in the Process of Hepatocellular Carcinogenesis

3.1. Properties of enzyme-altered foci.

Enzyme-altered foci are postulated as representing a stage of hepatocellular carcinogenesis in rats that precedes the tumour. An enzyme-altered island may constitute a clone of cells derived from a single hepatocyte that has undergone a mutation. Alternatively, it may be no more than a clone derived from a progenitor cell that has undergone a permanent phenotypic change (cf. a clone of a thymus-derived lymphocyte that has responded to the presence of an abnormal protein on the surface of a somatic cell). On current evidence, the development of enzyme-altered foci is not thought to be obligatory in the development of hepatocellular carcinomas.

Gössner and Friedrich-Freksa(1964) observed glucose-6-phosphatase (G-6-P)-free areas in the liver sections of rats continuously given diethylnitrosamine. They called these "islands" (Friedrich-Freksa et al.,1969 a-b), and such islands were later observed during similar experiments (Schauer and Kunze, 1968) in liver sections stained for nucleoside-5'-triphosphatase (ATPase). Subsequently it became clear that strong hepatocarcinogens, including diethylnitrosamine, dibutyl-nitrosamine, acetaminofluorene, 1,2-dimethyl-hydrazine and N-nitro-somorpholine, induced such foci within only a few weeks when continuously fed to rats (Friedrich-Freksa et al.,1969 a-b); Rabes et al.,1972; Schieferstein et al., 1974; Scherer and Emmelot 1975; Kitagawa, 1971; Kunze et al.,1970; Taper et al.,1971). Not only were enzyme changes with respect to loss of G-6-P and ATPase observed, but also foci of diminished glycogen phosphorylase and elevated arylesterase and γ-glutamyl-transpeptidase (γ-GT) were also reported. Combinations of such different lesions are frequently observed since a single focus may show several of these changes (Schieferstein et al.,1974; Scherer and Emmelot,1976; Sirica et al.,1978).

The mechanism which leads to phenotypic heterogeneity of the enzyme-altered foci is still not known. It has been suggested, but not proven, that the phenotypic heterogeneity of hepatocellular carcinomas may be due to alterations in m-RNA template stability (Pitot et al.,1974) and that a similar mechanism could operate in enzyme-altered foci (Sirica et al.,1978).The size and quantity of these foci correlate quantitatively with the amount of the hepatocarcinogen and the duration of its action on the organism (Scherer and Emmelot,1976; Kunz et al.,1976; Emmelot and Scherer,1977). Kunz et al.(1978) demonstrated that the formation of

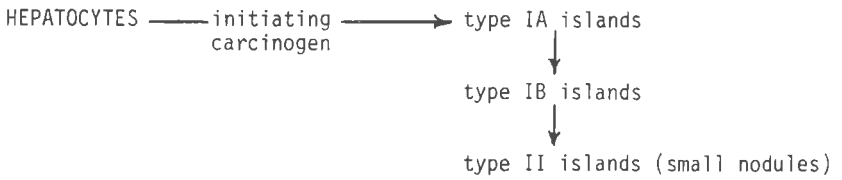
ATPase-deficient foci precedes hepatic tumour formation but obeys the same quantitative laws of dose/time dependence (Druckrey et al.,1963) as are known for tumourigenesis.

An enhancement of the sensitivity of hepatocytes in responding to a carcinogenic stimulus with island formation can be achieved by using very young or newborn rats (Laib and Bolt,1980; Peraino et al.,1981), or by partial hepatectomy (Laib and Bolt,1980; Pitot,1979). Thus, vinyl chloride and some related compounds, which in adult rats produce only hemangioendotheliomas of the liver, cause the formation of hepatocellular carcinomas in newborn rats (Maltoni,1977) and the latter is preceded by the formation of enzyme-altered foci (Laib and Bolt,1980).

So far, studies on enzyme-altered foci as precursor stages of hepatocellular tumourigenesis have been carried out most extensively in the rat. Data on other species have shown that guinea pigs developed ATPase-deficient foci after diethylnitrosamine treatment but to a lesser degree than did rats, whereas several strains of mice did not respond in this way (Laib et al.1982). However, mice neonatally treated with diethylnitrosamine developed foci deficient in G-6-Pase (Moore et al.,1981). It has not been definitively shown whether enzyme-altered foci occur in human liver (Kalengayi,1978).At present, attempts are being made to use the quantification of enzyme-altered foci in rat liver in defined experimental schedules to identify tumour promotors (Schulte-Hermann et al.,1981; Tennekes et al.,1981) and to compare the activity of known hepatocarcinogens and mutagens in the experimental induction of such lesions in the rat (Tatematsu et al.,1977; Laib and Bolt,1980).

3.2. Stages of development

Scherer and Hoffmann (1971) proposed a clonal origin of enzyme-altered foci. They demonstrated that on simultaneous administration of ^{3}H-thymidine and 10 mg/kg diethylnitrosamine to 2/3-hepatectomised rats, the ^{3}H-thymidine labelling was lost from the foci after 5 weeks. This was in contrast to the surrounding hepatic parenchyma which was heavily labelled. It was suggested that repeated cell divisions in the focal area had caused dilution of the radioactive marker. This view is also consistent with later publications (Emmelot and Scherer,1980). Recently, the following tentative sequence has been proposed by Emmelot and Scherer (1977) :



The first type (IA) islands, which are small (up to 200 µm in diameter), appeared up to 6 weeks after diethylnitrosamine administration and were situated mainly between the central vein and the periphery of the liver lobule. The cells were characterised by a complete loss of bile canalicular ATPase and a marked decrease in cytoplasmic ATPase. Some were also deficient in G-6-Pase (Scherer and Emmelot,1976).

According to Kuhlmann et al.(1981) another sensitive marker for very early enzyme changes is epoxide hydrolase which increases as ATPase decreases. In such early lesions γ-GTase is not altered.

The later-appearing (IB) islands are larger and are found more in the central area of the liver lobule. An internal heterogeneity of marker expressions seems to be characteristic of this type of island (Emmelot and Scherer,1980).

At the type II stage (small nodules with a diameter of at least 1 mm) the coincidence of ATPase and G-6-Pase deficiencies is markedly enhanced and all the ATPase lesions are also glycogen positive.

3.3. Relation of enzyme-altered foci to hepatocellular cancer

There is now a body of data suggesting a relation between foci and cancer but it is not clear how the alteration in one or more distinct enzymes is mechanistically connected with the process of hepatocarcinogenesis. Emmelot and Scherer (1980) recently listed all the qualitative and quantitative arguments in support of a relationship between islands and hepatocellular tumours, namely : that precancerous cells share some common features with tumour cells; that precancerous cells precede tumour cells in the sequence of events; and that tumour cells can be demonstrated to be in close association with precancerous lesions.

Another question is that of the spontaneous appearance of enzyme-altered foci in rat liver. This may vary in quantity from strain to strain, but spontaneous islands can generally already be observed in rats at 18-25

weeks of age (Ogawa et al., 1981). Although the reason for this phenomenon is not known, environmental, dietary and genetic factors may be involved . Also, it is not known why young female rats are more susceptible to the formation of ATPase-deficient islands than are male rats (Laib et al.,1979; Deml et al.,1981). This contrasts with the higher incidence of tumours in male rats.

The appearance of islands is influenced by genotoxic and non-genotoxic effects on the liver cell. For instance, phenobarbital at a dose of 75 mg/kg per day significantly raised the number of ATPase-negative and γ-GT-positive islands in livers of rats pre-treated with the genotoxic carcinogen N-nitrosomorpholine (Hacker et al.,1981). For discrimination between genotoxic and non-genotoxic (promotional) effects the use of very young rats may be advantageous since they are very sensitive towards initiating hepatocarcinogens and do not show spontaneous islands (Deml et al.,1981).

In general, the whole area of preneoplastic enzyme-altered foci is under study in many current research programmes, and new evidence should become available in the near future to clarify their role in the process of hepatocellular carcinogenesis.

E. DISTINGUISHING FEATURES OF GENOTOXIC AND NON-GENOTOXIC MECHANISMS IN HEPATOCARCINOGENICITY

Throughout this monograph the importance of the distinction between genotoxic and non-genotoxic mechanisms in the genesis of liver neoplasia has been noted frequently. It is emphasised that it is not always easy to allocate a particular chemical unequivocally to one or other of these categories. These features should be considered only as a reflection of the current scientific thinking about carcinogenesis, and of the quality and sensitivity of the test methodology used. Other aspects of the toxicology of the material under question must receive due consideration in making an assessment of carcinogenic potential. In this chapter the features of genotoxic and non-genotoxic agents are compared and contrasted, and some of the important characteristics that distinguish genotoxic from non-genotoxic hepatocarcinogens are presented in Table 2.

Table 2

Features for Distinguishing Genotoxic and Non-genotoxic Mechanisms in Hepatocarcinogenesis

Genotoxic Mechanisms	Non-Genotoxic Mechanisms
1) Reactivity of chemical and/or metabolites with genetic material predictable	1) No obvious chemical reactivity predictable
2) Positive results in appropriate mutagenicity tests	2) Negative results in mutagenicity tests
3) Evidence of interactions with DNA	3) No evidence of interaction with DNA
4) No apparent dose-response threshold	4) Threshold may be apparent
5) Organs other than the liver may be affected	5) Liver is normally the only organ affected
6) Effect evident in more than one species	6) Effects confined to a single species or to rats and mice only.
7) Broad spectrum of histopathological types of liver tumour	7) Narrow spectrum of histopathological types of liver tumour
8) Hepatotoxicity not essential	8) Hepatotoxicity usually evident
9) Partial hepatectomy enhances the effect.	9) No enhancement by partial hepatectomy.

1. Chemical Structure, Reactivity and Metabolism

It may be clear from the structure of a chemical that it is itself electrophilic or that it is likely to be metabolised to an electrophile in vivo. In the former case, the tissue most at risk is likely to be the first one that comes into contact with the agent and the likelihood that other tissues, such as the liver, will be at risk depends on factors such as the chemical half-life of the agent and the extent to which it reacts with macromolecules on first impact. Where the chemical requires metabolic activation, the tissues most at risk are those where this occurs.

Structural considerations may help in chosing suitable experimental designs, e.g. it is important that the chemical class or structural group to which a substance belongs should be taken into account in deciding which short-term screening tests should be used.

2. Mutagenicity

Positive results in bacterial mutation screening tests (base-pair substitution or frameshift mutation) in the absence of the liver-derived metabolizing enzymes, suggests that an agent is electrophilic or is transformed into an electrophile by bacterial enzymes. Positive results in such tests after the addition of liver-derived enzymes suggest that the chemical is metabolised to an electrophile. In addition, positive results in mutation tests, including in vitro cytogenicity in mammalian cells, in vivo tests for point mutation and in vivo tests for chromosome damage, would indicate that a substance is a acting by a genotoxic rather than a non-genotoxic mechanism.

3. DNA Interactions

The most scientifically-satisfying evidence that an exogenous chemical has interacted covalently with DNA is the demonstration, by biochemical techniques, of the formation of DNA adducts (cf.Appendix 2).

4. Threshold Level

Dose-response data presented for a range of genotoxic and non-genotoxic compounds (Newberne, 1965 ; Druckrey et al. 1963 ; Peto et al., 1982 ; Tomatis et al., 1972 and 1974; Walker et al. 1973 ; Ikeda et al. 1966 and 1968) indicate that differences exist between the nature of the relationships obtained and the dose levels utilised. It must be realised, however, that in a screening assay to determine whether a substance is carcinogenic, only a limited number of doses are used and the exact nature of the dose response dependency is not clearly identified. The genotoxic

substances (Newberne, 1965 ; Druckrey et al., 1963 ; Peto et al. 1982) exhibited a very clear increase in tumorigenic response with increasing dose over a wide range of dose levels (ppb to ppm). These examples, however, were derived from experiments designed specifically to characterise the response exactly. The non-genotoxic examples cited (Tomatis et al., 1972 and 1974 ; Walker et al., 1973 ; Ikeda et al, 1966 and 1968) give a step function response, probably due entirely to the limited dose range used. Nevertheless, a common feature of these data is the comparatively high dose levels needed (100's ppm) before any effect above background was observed. This does not necessarily mean, however, that a threshold exists before a carcinogenic response is obtained. To clearly identify any meaningful differences in the shape of the dose-response curves for the genotoxic and non-genotoxic substances it will be necessary to use a larger number of dose levels in the experiments.

5. Specificity of Tumour Sites

Although ionizing radiation and certain genotoxic carcinogens are capable of causing cancers in a variety of tissues, for many non-genotoxic carcinogens other than hormones the liver is the usual target organ. Thus, if an agent which gives rise to liver tumours also gives rise to tumours of one or more specific types, then it is more likely to be acting by a genotoxic than a non-genotoxic mechanism.

6. Specificity of Species

In general, an agent which produces tumours of one or more types in more than one animal species is more likely to be a genotoxic carcinogen than is an agent which gives rise to only one kind of tumour in a single species. Hence, if after adequate carcinogenicity studies in several species the only positive effect noted is an increase in the incidence of liver tumours in,say, the mouse, then a non-genotoxic mechanism should be seriously considered. By this type of study alone a genotoxic mechanism cannot be totally excluded since there can be both quantitative and/or qualitative differences in biotransformation between different species.

7. Spectrum of Liver Tumours Arising

A feature of potent genotoxic hepatocarcinogens is that tumours are commonly produced in a variety of cell types . The spectrum often includes both bile-duct and hepatocellular neoplasms, and sometimes tumours derived from the hepatic vascular structures and reticulo-endothelial elements (i.e. Kupffer cells). In contrast, the spectrum of tumours seen in response to non-genotoxic carcinogens is characteristically narrow,

usually affecting the hepatocyte series only. Also, the response to genotoxic carcinogens is often the occurence of highly malignant tumours , whereas this is less often the case with non-genotoxic carcinogens.

8. Significance of Hepatotoxicity

Hepatotoxicity is a broad term encompassing a variety of changes. At one end of the spectrum, changes in hepatic function (e.g. as indicated by an increase or decrease in the levels of particular liver enzymes) may be regarded as evidence of toxicity even though this may constitute no more than reversible adaptive changes. At the same end of the spectrum are increases in liver size. At the other end of the spectrum can be found liver cell necrosis and/or cirrhosis, often associated with nodular hyperplasia.

It is probable that chronic toxicity, particularly that associated with fibrosis, is invariably an indication of an increase in liver tumour risk although it is clear that the extent of the increased risk varies widely , depending on the cause of the cirrhosis. At higher dose levels most genotoxic hepatocarcinogens cause toxic effects in the liver. However, it is claimed that at least some genotoxic hepatocarcinogens at low doses can lead to liver tumours without overt evidence of toxic change. In some cases this claim may be unwarranted insofar as no one has looked for evidence of toxicity at the right time. By contrast, the distinguishing feature of a non-genotoxic carcinogen is that its activity is associated with long-lasting changes in liver morphology and biochemistry.

9. Effect of Partial Hepatectomy and Promotors

The role of partial hepatectomy in differentiating between genotoxic and non-genotoxic mechanisms has been discussed above, in chapter C.3.4.In the field of initiating-promoting assays there is much current work aimed at getting a better insight into the various mechanisms of hepatocarcinogenesis (Williams,1981). If validated, these assays may be valuable tools for differentiating between genotoxic and non-genotoxic mechanisms.

10. The Significance of Background Liver Tumour Incidence.

As pointed out above, the distinction between genotoxic and non-genotoxic mechanisms may be blurred because of interactions between the effects of the tested chemical and background factors. Examples of background factors that might be implicated in this way are discussed at length in chapter C. In particular, the influence of hormonal status, dietary fat intake and

nutritional status on the incidence of spontaneous liver tumours in rodents must be fully taken into account when interpretating laboratory studies. It is necessary to explore the possibility that a difference in liver tumour incidence between exposed and unexposed animals depends on a difference in one or more of these non-specific factors, rather than on exposure to the test chemical. Furthermore, since the conditions under which virtually all long-term experiments on rodents are nowadays performed predispose to high incidences of endocrine disorders, including neoplasms of endocrine glands and hormone-dependent tissues, such animals are far from ideal models for investigating the potential carcinogenicity of chemicals in hormonally-normal humans. The marked sex difference in the incidence of spontaneously-occurring liver tumours in mice indicates that this type of tumour is influenced by the sex-hormone status. Because of these considerations one should not automatically conclude that enhancement of the incidence of liver tumours in rodents implies that a chemical is acting by a genotoxic mechanism.

General cancer theory suggests that the first stage in carcinogenesis is necessarily of the nature of a mutation and that non-genotoxic mechanisms do no more than supplement the effects of mutagens.This concept is either wrong or without meaning, particularly in the case of strains of laboratory mice that exhibit a high spontaneous incidence of hepatocellular tumours. In such strains one needs to postulate that a genetic flaw serves as the tumour-initiating event. The unanswerable question then arises : to what extent are similar genetic flaws affecting the liver to be found in other strains of mice and other species, including man? It is partly because this question cannot be answered that the possible risk to man of non-genotoxic agents has to be considered seriously. In this light, a high incidence of mouse-liver tumours might be seen as no more than a sensitive tool for the detection of non-genotoxic carcinogenicity. However, in practice its value is extremely limited because non-specific factors, particularly sex hormone status, endocrine status generally, and nutritional status are known to be major determinants of tumour incidence.

Potent genotoxic hepatocarcinogens give rise to liver tumours in strains of mice exhibiting both high and low incidences of spontaneous liver tumours. Generally, a non-genotoxic mechanism is to be suspected if enhancement of liver tumour incidence is greater in mouse strains with a high background incidence, and/or is much more evident in males than in females.

F. COMPARATIVE ASPECTS OF ANIMAL AND HUMAN HEPATIC NEOPLASIA

A variety of hepatic tumours occur both in animals and man. In this monograph, attention has been directed principally to hepatocellular tumours, although tumours originating from non-parenchymal cells have been referred to where necessary to illustrate important points in the mode of action of chemical carcinogens.Despite some differences in morphology, hepatocellular tumours both in man and animals exhibit the biological characteristics of benign and malignant tumours. Malignant tumours vary in biological behaviour and morphological structure, from differentiated slow-growing tumours to highly anaplastic rapidly-growing ones.

In addition to frank neoplastic lesions, a range of proliferative lesions which appear to predispose to, or to be involved in, the development of cancer can be found in man and animals. Although there is little disagreement among pathologists in identifying these lesions in man, there is considerable disagreement on the classification of nodular proliferative lesions in rodents, particularly in the mouse (cf.chapter C-1).

1. Aetiology of Hepatic Neoplasia in Man and Animals

1.1. Hepatitis B (HBV)

Hepatic neoplasia in man varies widely in geographical incidence. In certain regions of Africa and Asia there is a much higher incidence of liver cancer than in Europe and North America. This is associated with a high incidence of the hepatitis B infection where the chronic carrier state often leads to macronodular cirrhosis, this being the cirrhosis most frequently underlying human hepatocellular carcinoma. Compelling evidence for the carcinogenicity of the hepatitis B virus has arisen from studies indicating that there is covalent integration of the hepatitis B virus genome into the DNA of each of eight hepatocellular carcinomas (HCC) in HBV carriers (Shafritz and Kew,1981; Prince,1981).

In addition, when the mother is a carrier of HBV the relative risk of the child developing HCC is 15, and when the father is negative for HBV surface antibody the relative risk is 59 (Larouze et al., 1976). These data suggest that maternal transmission is an important factor and that there may also be an immunological defect inherited from the father. It is noteworthy that a remarkably apt animal model for HBV-related carcinoma has been recently reported, i.e. the woodchuck, which develops chronic viral hepatitis and HCC when infected with a virus closely resembling HBV-virus (Summers,1981 ; Johnson and Williams, 1982).

1.2. Cirrhosis

It has long been recognised that there is an association between HCC and cirrhosis. The proportion of HCC's with cirrhosis varies from 80 to 95% in different geographical areas. The frequency with which cirrhosis is complicated by HCC is strikingly different in areas of high and low incidence of the latter. In Chicago only 5% of patients with cirrhosis develop HCC (Stuart,1965) whereas in South Africa the figure for non-Caucasian Africans is between 40 and 50% (Thompson,1961). The Caucasian South African population has the low rate of Europeans. This suggests that in non-Caucasian Africans another factor in addition to cirrhosis is involved in the causation of HCC.

The predominant type of cirrhosis in each racial group may be relevant,eg. the majority of investigators have found that HCC occurs particularly in livers exhibiting the macronodular type of cirrhosis (Anthony,1976; Lee,1966; Shikata,1976). This form of cirrhosis is not usually associated with alcoholism but is, however, the most common form in areas where there is a high-frequency of HCC. An exception is that among non-Caucasian Africans in Southern Africa siderotic micronodular cirrhosis is the most common form (Shonland et al.,1979). In this study HCC was found to occur selectively in patients with siderotic livers and no fibrosis, suggesting that iron deposition was a factor in causing HCC independently of cirrhosis. It is perhaps also relevant that an increased incidence of HCC has been described in patients with inherited haemochromatosis, and in Africans with the acquired form (Warren and Drake,1951). No appropriate animal model of macronodular cirrhosis is available (cf. 3 of this chapter).

1.3. Other factors

In addition to cirrhosis and HBV, a number of ingested carcinogens have been proposed as aetiological factors.

1.3.1. Aflatoxin B_1

This is a potent hepatotoxin in both man and animals (Nayak and Ramalingaswahi,1978; Wogan et al.,1974) and has been suggested as an aetiological factor for HCC in man. The supporting data primarily concern areas of the world in which high incidences of HCC are observed (Linsell,1978). It has been suggested that the occurrence of human HCC is dose-dependent. Lutwick (1979) recently postulated that aflatoxin in man may not act as a primary carcinogen but as a suppressant of cell-mediated immunity, allowing the persistence of HBV infections. However, Primack et al.(1973) were unable to demonstrate

immunological suppression in Ugandan cases. Thus, if immune suppression plays a part in HCC it would seem to be an inherited or environmental suppressant, specific for HBV infection.

1.3.2. Steroids

There is a well-documented link between the occurrence in humans of liver-cell tumours (usually benign but occasionally malignant) and the use of contraceptives consisting of oestrogens and progestogens (Baum et al.,1973; Neuberger et al.,1980; Klatskin, 1977). Although less well documented, there appears to be a clear link between excessive exposure to androgens (usually oxymethalone or methyltestosterone) and an increased risk of liver cell tumours in humans (Bernstein et al.,1971; Johnson et al.,1972; Farrell et al.,1975; Sweeney and Evans,1976).

When laboratory mice were administered several contraceptive-pill formulations of oestrogens and progestogens, the incidence of liver cell tumours exceeded that in untreated animals (Committee on Safety of Medicines,1972). As indicated above (chapter C.3.2.1.) male rats and mice are more susceptible to liver tumour development than are females (Toh, 1973).The reduction in liver tumour incidence in male rats following castration can be reversed by administering testosterone (Firminger and Reuber, 1961). Moreover, administering testosterone to either intact or gonadectomized male or female rats raises the liver tumour incidence (Firminger and Reuber, 1961), as does the administration of some progestational compounds to either intact male, or gonadectomised male and female rats (Reuber and Firminger,1961; Schuppler and Günzel,1979).

1.3.3. Other Chemicals

Vinyl chloride (Creech and Johnson,1974), thorium dioxide (Dahlgren,1961; Faber,1973;) and arsenic (Roth,1957; Popper et al.,1977) are proven human chemical hepatocarcinogens, producing neoplasms derived from non-parenchymal cells of the liver. These agents apparently have different mechanisms of action. Vinyl chloride is transformed into a reactive alkylating metabolite, thorium dioxide is thought to act via an ionizing-radiation mechanism, and arsenic probably inhibits DNA repair processes. (cf. chapter D).The data are insufficient for evaluating the carcinogenicity of thorium dioxide and arsenic in animals (Popper,1978), whereas vinyl chloride is an

example where the human liver cancer hazard has been adequately matched by animal studies (Maltoni and Lefemine,1974).

A wide range of compounds in different chemical classes has been shown to cause neoplasia in rodents. These findings have often resulted from the administration of doses close to the Maximum Tolerated Dose for the rodents' life-time, and the development of tumours is often preceded by the appearance of nodular hyperplasia. The compounds include some progestational agents reported probably to have produced liver cancer in man (Schuppler and Günzel,1979). Certain compounds have also produced tumours when administered at low doses, or for short periods, without the prior appearance of cirrhosis.

There is apparently a lack of good epidemiological data on established animal hepatocarcinogens. However, where such data do exist, ie. for compounds such as phenobarbital (Clemmesen and Halgrim-Jensen,1978,1981), clofibrate (IARC,1980), DDT (IARC,1974) and isoniazid (Jansen et al., 1980; Howe et al.,1979; Costello and Snider,1980) no excess liver cancer hazard to man has been observed. These compounds are considered to be non-genotoxic carcinogens in rodents. Certain genotoxic compounds which produce liver tumours in rodents have not elicited hepatic carcinomas in man, but have led to tumours at other sites (e.g. β-naphthylamine and benzidine). However, there is no example of a compound believed to act by a non-genotoxic mechanism in rodent liver which is a proven human chemical carcinogen at a different site.

This situation emphasises the need for a thorough understanding of the mode of action of the chemical in the rodent and for a comparison of this with its biological properties in man or, if this is impossible, in another primate.

2. Non-neoplastic Proliferative Lesions in Man and Rodents

Two types of nodular lesion in man which were in the past suspected of possessing neoplastic potential are now considered as probably having no such potential. The first type is focal nodular hyperplasia which occurs (but only very rarely) in young women (Lough et al., 1980). The second type, also a rare condition, is characterised by multiple regenerative nodules which resemble those of cirrhosis but are not separated by connective tissue (Knowles et al.,1975). Neither of these lesions appear to progress to

malignancy but it can be argued that the information is inadequate for this to be certain.

Cirrhosis in rodents has not been reported as a naturally-occurring disease (Koo et al., 1980). A few chemicals, e.g. carbon tetrachloride, produce liver lesions that satisfy certain morphological criteria of cirrhosis. The model is of limited relevance because the regenerative capacity of the rodent liver cannot be compared to that of man.

The solitary hyperplastic nodule in the rodent bears some resemblance to focal nodular hyperplasia in man, while the multiple nodularity without fibrosis, sometimes produced by chemicals in rodents, resembles the multiple nodular hyperplasia in man. Experience so far seems to indicate that some, at least, of these proliferative lesions in the rodent progress to carcinoma, and indeed some pathologists regard them as being of neoplastic origin.

G. SEQUENCE OF STEPS RECOMMENDED FOR ESTABLISHING THE HEPATOCARCINOGENIC POTENTIAL OF A CHEMICAL TO LABORATORY ANIMALS AND MAN

The detection of hepatocarcinogenicity relies on long-term studies in animals. Although there are no reliable short-term tests that relate specifically to the detection of hepatocarcinogenicity, an early indication of possible hepatocarcinogenicity, or the lack of it, can be deduced from the results of other tests. Thus, a compound is unlikely to be a hepatocarcinogen if it gives negative results in mutation tests and no liver enlargement or disturbance of liver micro-architecture in an appropriate 14-day rat study. More substantial evidence is the failure to observe adverse hepatic changes in a 90-day rodent study. If liver changes are present in either a 14- or 90-day experiment, they may require further investigation.

1. Stepwise Approach to the Investigation of Possible Hepatocarcinogenicity

The sequence of steps is summarised in Tables 3 and 4. It is emphasised that this guidance scheme should not be interpreted rigidly. The investigations carried out will vary from chemical to chemical, and many factors (e.g. physico-chemical properties, potential routes of exposure) may influence the choice of experimental systems.

As discussed in chapter F, the first three types of information concern chemical reactivity, genotoxicity and short-term *in vivo* toxicity studies for identifing possible target tissues and demonstrating the presence or absence of cumulative toxicity. In the light of this information one of the following four positions may be reached (see also Table 3) :

- mutation negative, liver changes absent
- mutation positive, liver changes absent
- mutation positive, liver changes present
- mutation negative, liver changes present

When mutagenicity tests are negative, and there is no evidence of hepatotoxicity in short-term tests, then no priority need be given to the investigation of hepatocarcinogenicity.

If mutation tests are positive but there is no evidence of liver toxicity, the next step is to seek confirmation of the mutagenic properties (ECETOC, 1980), possibly extending the search to an investigation of DNA-adduct formation and the nature and response of DNA repair processes. If these

mutation tests confirm that the chemical has mutagenic potential, further studies (possibly including long-term animal studies) relevant to possible human exposure will usually be required to assess the carcinogenic potential for organs other than the liver.

If the mutation tests are positive and there is evidence of liver toxicity in short-term tests, corroborative tests for mutagenicity are required (ECETOC , 1980) and short-term toxicity studies extending to other animal species are advisable. These should include interspecies comparative studies to resolve possible variations in qualitative and/or quantitative metabolism and detoxification, including investigations in a non-rodent species. This would aid in the differentiation of species-specificity regarding hepatic response. It should not be assumed at this stage that there is any relationship between positive findings in the mutagenicity tests and hepatotoxicity.

If the mutation tests are negative and liver toxicity positive, attempts should be made to confirm non-genotoxicity using a relevant in vivo procedure (ECETOC 1980). The nature of the hepatotoxicity should also be characterised. Possible observations may include classical histopathological changes (e.g. zonal/focal degeneration) in the absence of liver enlargement, in which instance the compound is a hepatotoxin and attempts at determining no-effect levels should be made. Alternatively, liver enlargement may be observed in the absence of histopathological changes, and in this situation attempts should be made to differentiate between liver cell enlargement per se and cell proliferation (see chapter D). Studies such as thymidine incorporation, estimation of ploidy, and counting of mitotic figure are useful in differentiating between the two processes. In many cases both cell enlargement and cell proliferation may be observed. Where cell proliferation is encountered, the use of other rodent or non-rodent species should be considered to give a better assessment of potential human hazard.

Cell enlargement is frequently encountered and can be detected by microscopic or biochemical (DNA concentration) procedures. Factors often responsible for liver cell enlargement are increased intracellular lipid, or the proliferation of peroxisomes and smooth endoplasmic reticulum. Fatty infiltration may be determined histochemically, while proliferation of subcellular organelles may be measured either ultrastructurally or biochemically. Peroxisome proliferation may at times be preceded by fatty change of the liver, and hence if lipid accumulation is observed one might look carefully for peroxisome proliferation at a later stage.

There seems to be little, if any, causal relationship between the proliferation of smooth endoplasmic reticulum (SER) and hepatocarcinogenicity. Conversely, there seems to be a reasonably good correlation between the ability of a chemical to elicit peroxisome proliferation and the subsequent appearence of hepatic tumours in rodents. To assess the significance of such observations for man, short-term *in vivo* tests in a non-rodent species should be considered.

2. The Importance of Comparative Studies of Metabolism and Pharmacokinetics

Much of the above is relevant to the question of extrapolation to man. However, the most important information for genotoxic and non-genotoxic carcinogens alike comes from comparative studies of metabolism and pharmacokinetics. In the biotransformation of exogenous chemicals there can be important qualitative as well as quantitative differences between species and it is essential in assessing the possibility or extent of adverse effects in man, to look for such differences and take account of them.

TABLE 3
Sequence of Steps - I

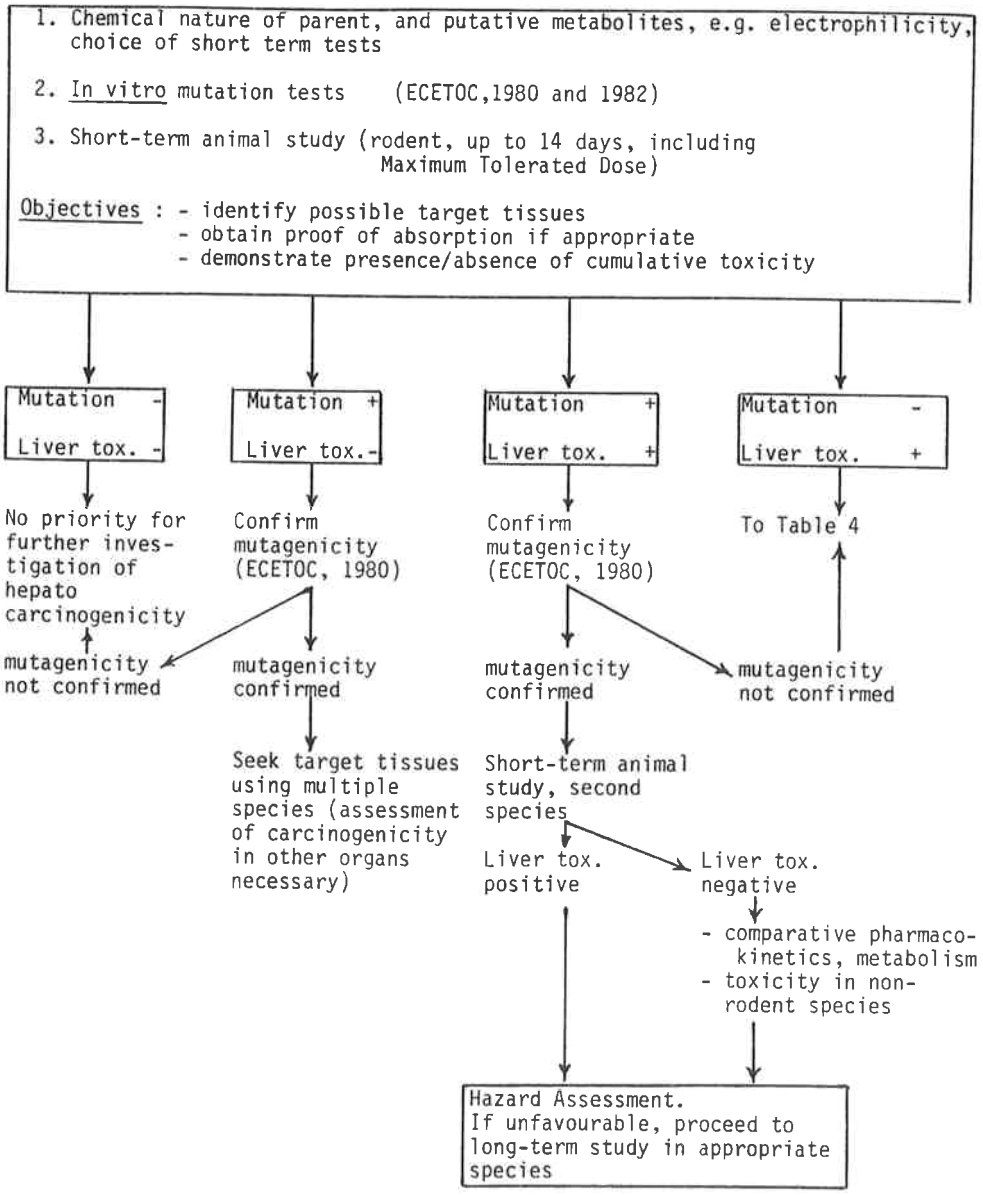
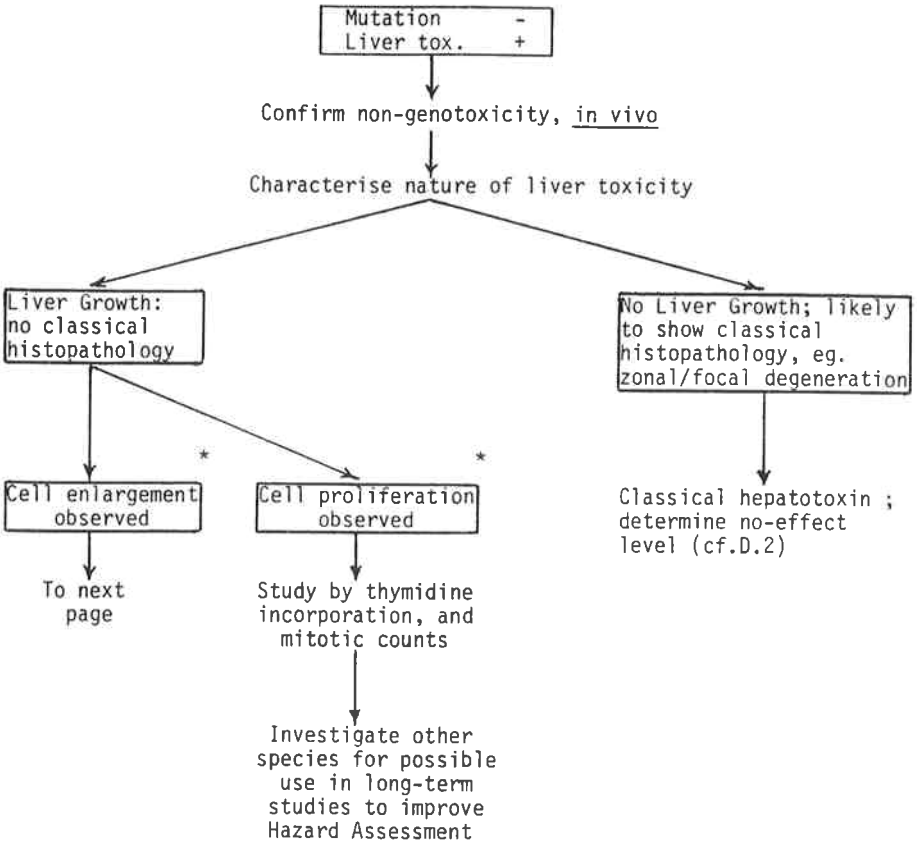


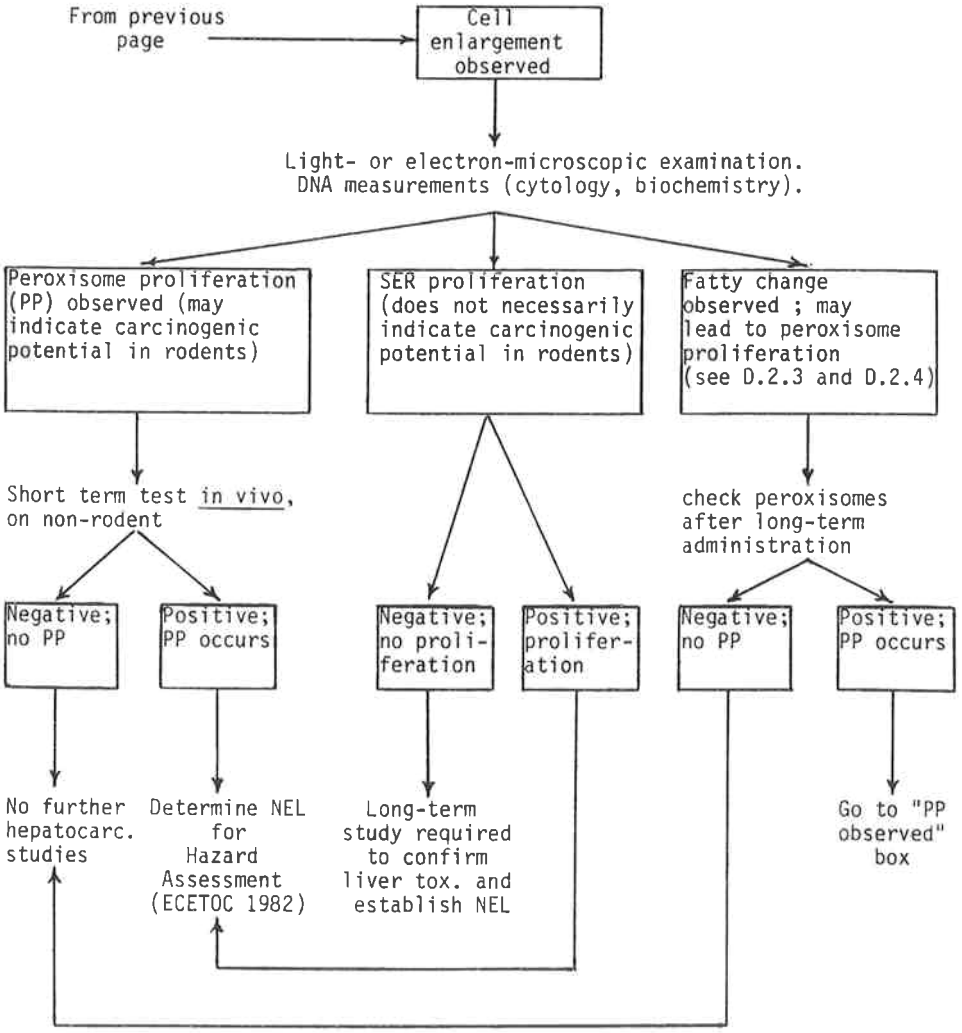
TABLE 4

Sequence of Steps-II



*Both effects may appear simultaneously.

TABLE 4 (continued)



H. AREAS DESERVING FURTHER INVESTIGATION

In preparing this monograph the Task Force perceived a lack of information in several areas relating to hepatocarcinogenesis in the experimental models and in humans. In particular, while much scientific effort has been expended on the study of genotoxic mechanisms, data about the mode of action of non-genotoxic agents is scarce. It is against this background that certain topics, identified below, appear to merit further investigation.

1. The significance of enzyme-altered foci (islands) in the pathogenesis of liver cell neoplasia needs to be elucidated.

2. A better understanding of the mechanisms and processes involved in the non-genotoxic enhancement of liver tumour incidence is needed.(e.g. liver growth, ploidy changes, peroxisome proliferation).

3. Further research is needed into the role of dietary factors, nutritional status and stress in the incidence of spontaneous and chemically-induced liver tumours.

4. The relationship between nodular hyperplasia, benign neoplasia and malignant neoplasia of the liver requires further study .

5. Data relevant to the comparison of responses to foreign compounds in man and various animal species is needed, e.g. utilizing primary hepatocyte culture systems from a range of laboratory animals and man.

I. SUMMARY AND CONCLUSIONS

1. Liver tumours are common in untreated laboratory rats and mice and are readily produced in these species by exposure to a wide variety of chemicals. By contrast, they are rare in Western man. Hepatitis B virus is implicated in the aetiology of many human liver cancers, whereas no comparable virus has been identified in rats, mice, hamsters or most other species. Similarities and contrasts between man and other species are discussed in chapter E.

2. Many factors influence the spontaneous incidence of liver cell tumours in laboratory animals. Genetic factors are important as determinants of the widely-differing incidences encountered in mice of different inbred strains. Dietary levels of fat, the amount of food consumed and sex-hormone status head the list of other important determinants of spontaneous tumour incidence. In the interpretation of liver tumour data from long-term animal studies, and in the approach to testing chemicals for potential hepatocarcinogenicity, it is essential to take these background considerations into account.

3. Liver tumour incidence may be enhanced either by exposure to genotoxic mutagens or to certain non-genotoxic agents. The former type of activity is generally a matter of greater concern than the latter because it is frequently difficult to identify any reason why low levels of exposure should not carry proportionately low levels of risk. By contrast, for agents acting by non-genotoxic mechanisms and enhancing liver tumour incidence under conditions of high exposure, it is often possible to identify convincing reasons why there should be no risk under conditions of low exposure.

4. Throughout the present monograph, therefore, considerable importance is attached to the distinction between genotoxic and non-genotoxic mechanisms. Most hepatocarcinogens are derived by the metabolic activation *in vivo* of non-carcinogenic precursors, usually as a consequence of specific enzymatic activity. Species and strain differences in the enzymatic activity involved partly account for differences in susceptibility to liver tumour development in response to certain agents. The implications of the need for metabolic activation in relation to genotoxic and non-genotoxic mechanisms are discussed in chapter B.

5. Chemicals which induce liver tumours by a genotoxic mechanism are usually, if not always, unequivocally mutagenic in a variety of appropriate *in vitro* and

in vivo tests. They form demonstrable adducts with DNA and often induce unscheduled DNA synthesis. Furthermore, they elicit a clear dose-response relationship in appropriately-designed studies and can in many instances induce tumours at levels below hepatotoxic doses. The tumours may range from the well-differentiated to the poorly-differentiated, and may originate not only from hepatocytes but also from other cell types present in the liver (e.g. bile duct; endothelial and Kupffer cells). By contrast, compounds which give rise to liver tumours by non-genotoxic mechanisms are either not mutagenic or elicit an equivocal result in such tests, particularly in *in vitro* tests. DNA repair is not induced, and no adducts are formed with DNA. Some hepatotoxic response is usually seen at dose levels which produce tumours, but not at lower levels. Liver injury may consist, at one extreme, of hepatocellular necrosis and at the other it may involve changes which are definable only ultra-structurally or biochemically (e.g. peroxisome proliferation or autophagy formation).

6. A systematical approach to the evaluation of a chemical for hepatocarcinogenic potential for man is outlined in chapter G.In evaluating the significance of hepatic tumour induction in rodents the first step is to seek to eliminate the possible extraneous factors contributing to the increased tumour incidence. The next step is to investigate the mechanism of action and the pharmacokinetics at low and high dose levels in rodents. It is helpful if some knowledge of the pharmacokinetics of the compound in man is available. In assessing potential hazard to man, greater weight must be given to evidence suggesting a genotoxic mechanism but it should not be assumed that an agent that enhances the incidence of liver cancer in rodents by a non-genotoxic mechanism poses no risk for humans.However, in assessing the potential hazards of a non-genotoxic carcinogen tested at a Maximum Tolerated Dose for prolonged periods of time, the consequences of such sustained perturbations in the metabolism of the animal should be considered. It is possible that what is initially a beneficial biological adaptation may eventually become detrimental because of concomitant alterations in metabolism and its regulation.

7. Throughout the monograph it is emphasised that hepatocarcinogenesis is merely one aspect of carcinogenesis. It is important to stress this because an agent that predisposes to liver cancer in one species, or under one set of circumstances, may predispose to cancers at other sites in other species or circumstances.

8. The monograph concludes with a list of areas deserving further investigation. Prominent in this list is the need for a better understanding of the significance of enzyme-altered foci (islands) and ploidy in relation to hepatocarcinogenesis; of the role of diet, nutritional status and stress as determinants of liver tumour incidence; and of the mechanisms underlying liver enlargement and overt differences in response between various species.

J. GLOSSARY OF TERMS

The terms defined here are mostly those which are sometimes given different interpretations by different authors.

CELITE^{R} : Trademark for diatomaceous earth and related products.

ENZYME-ALTERED FOCI (sometimes referred to as islands): lesions smaller than a lobule that display different cytochemical phenotypes. Lesions are spontaneous or chemically induced.

EOSINOPHILIC CYTOPLASMIC INCLUSION: a discrete structure which stains more intensively red than the surrounding cytoplasm.

GENOTOXIC : this term implies that a chemical (or a metabolite) interacts per se with DNA, or interferes with DNA replication or repair. Chemicals designated as genotoxic are generally mutagenic when tested by appropriate methods.

HAEMOCHROMATOSIS : a disorder of iron metabolism characterised by excess deposition of iron in the tissues, especially in the liver and pancreas.

HYPERPLASIA : the abnormal multiplication, or increase in number, of cells in tissue, or an increase in ploidy (cf. Appendix 1).

MONO-OXYGENASE : enzyme of the oxidoreductase class that catalyses the incorporation of one atom from molecular oxygen in the substrate.

NEOPLASM : a new growth of tissue in which the growth is uncontrolled and progressive. Malignant neoplasms are distinguished from benign in that the former show anaplasia (loss of differentiation) and have the properties of invasion and metastasis.

NON-GENOTOXIC : this is an operational term used to imply a mechanism of carcinogenicity whereby the chemical (or a metabolite) does not directly interact with DNA. Indirect effects upon DNA (increased mitosis, peroxidative damage, etc.) are included in this term.

PEROXISOME : a sub-cellular organelle containing oxidative enzymes, e.g. urate oxidase, amino-acid oxidase, catalase, and other enzymes.

PLOIDY : a general term refering to the number of chromosome sets per nucleus.

PROCARCINOGEN : a compound which requires metabolic activation before it can exhibit its carcinogenic potential.

PROXIMATE CARCINOGENS : compounds which are capable of producing cancer without prior metabolic activation.

XENOBIOTIC : a chemical foreign to the biological system.

K. APPENDICES

APPENDIX 1

POLYPLOIDY IN RODENT LIVER

1. General

Polyploidy can be regarded as an increase in the amount of genetic material of the cell as a consequence of the normal course of mitosis being blocked, or of an omission of certain phases in the mitotic cycle (Rudkin,1973). The increase in the genetic material is usually an exact multiple of the diploid state.

Polyploidy develops in cells which retain their ability to reproduce themselves despite the acquisition of specialised functions. During the process of cell proliferation, the specialised functions tend to be repressed but they return to normal as soon as the dividing cells revert to the resting phase (Brodsky and Uryvaeva,1977).There are three principal ways by which polyploid cells may arise in mammals :

a) Acytokinetic mitosis: a process involving division of the nucleus without an accompanying division of cytoplasm, and thought to be responsible for the production of binucleate cells in rodent liver. Each nucleus of the binucleate cell may be diploid or polyploid.

b) Endomitosis: abnormal forms of mitosis in mammalian tissues that give rise to polyploidy. The patterns chiefly encountered have been called C-metaphase, monopolar and multipolar mitoses, but other patterns of fusion of anaphase and telophase chromosomes have been described. These abnormal forms of mitosis are found in the liver of both man and rodents. In old rats they represent a significant proportion of all mitoses seen.It is thought that these abnormal mitotic figures represent an incomplete form of the mitotic cycle in which the chromosomes do not separate, but instead rearrange themselves into a cell nucleus of a higher polyploidy level.

c) Endonuclear reduplication: polyploidy arising by a blocking of the mitotic mechanism in which the cell cycle is arrested at the G_2 phase. The m-phase is then by-passed and the cell is able to proceed through a second, and further, cell cycles.Polyploid nuclei are able to divide, but they do so less frequently and at a slower pace than diploid nuclei (Sutou and Tokuyama,1974). They give rise to cells of the same ploidy level (Argyris,1971; Banerjee,1965).

2. Liver growth and polyploidy.

The liver of neonatal rats and mice consists principally of diploid cells. As the animals mature, the number of diploid (2n) cells gradually decreases and there is a corresponding increase of binucleate cells and cells with nuclei corresponding to 4n ploidy. This is followed by the appearance of nuclei of higher ploidy (Alfert and Geschwind,1958; Wheatley,1972) (Tables 6 and 7).

An acceleration in the development of higher ploidy levels occurs in the liver of rats and mice after partial hepatectomy (James et al.,1966), after induction of liver enlargement by certain agents that induce monooxygenase activity (Schulte-Hermann,1974) and after treatment with high doses of hepatotoxins (Christie and Lepage,1961; Hendy and Grasso,1977; Himes et al.,1957). An increase in ploidy levels is now considered to indicate hyperplasia (Barka and Popper,1967), so that the estimation of nuclear ploidy by biochemical or morphometric measurements may give valuable additional information on the state of the liver. It may also serve as an early index of tumour induction.

TABLE 6

Percentage of Diploid and Polyploid Cells in Liver of Young and Adult Mice (CBA/C57BL)

(Brodsky and Uryvaeva,1977)

	2n	2n.2*	4n	4n.2*	8n	8n.2*
Young (15-17g)	14	42	27	14	1	2
Adult (31-34g)	1	16	33	40	4	2

* binucleate cells

TABLE 7

Increase in Ploidy in Liver of Growing Rats

(Alfert and Geschwind,1958)

Age (days)	2n	2n.2*	4n	4n.2*	8n
14	90	5	5	-	-
21	70	25	5	-	-
28	55	35	8	2	-
43	43	35	20	2	-
61	33	35	28	4	-
90	17	18	55	8	2
114	15	10	60	10	5
365	5	2	65	13	15

* binucleate cells

APPENDIX 2

BIOCHEMICAL TECHNIQUES RELEVANT TO THE DISTINCTION BETWEEN GENOTOXIC AND NON-GENOTOXIC MECHANISMS

1. Introduction

Biochemical investigations can aid the differentiation between genotoxic and non-genotoxic mechanisms if a compound proves to be tumourigenic in animal bioassays. A definitive differentiation is not possible with one single method but requires consideration of various aspects.

Support for the hypothesis that a tumourigenic effect is caused by a non-genotoxic mechanism may emerge from: i) demonstration that the compound can enhance the incidence of liver tumours or the growth of preneoplastic hepatic enzyme-altered foci after previous "initiation" by a hepatocarcinogen; ii) characterization of the compound as non-mutagenic in short-term mutagenicity assays; and iii) biochemical studies at the DNA target level.For example, the following halogenated compounds do not induce mutations in the classical Salmonella/microsome assay (IARC,1979) although they are reported as producing hepatic tumours at high doses, at least in one species : chloroform, carbon tetrachloride, perchloroethylene, 1,1,2-trichloroethane (Weisburger,1977).

Biochemical studies at the DNA target level include the determination and identification of DNA alkylation products and studies of repair processes. For example, it has been demonstrated that no DNA alkylation occurs after application of chloroform (Diaz Gomez and Castro,1980-a; Reitz et al.1980) and also that no repair processes which could serve to indicate such lesions are induced (Reitz et al.,1980).

2. DNA Covalent-binding studies.

Because DNA covalent-binding studies require a complicated technique, completely divergent results have been obtained in different laboratories. Some workers have reported that carbon tetrachloride is transformed into metabolites that covalently bind to DNA of mice and/or rats (Rocchi et al., 1973; Diaz Gomez and Castro, 1980-b) whereas other investigators failed to confirm this (Harders et al.,1976; Harders,1976). Such discrepancies are due to differences in the purity of the radiolabelled compounds, and in the procedures for the isolation and purification of DNA. Contamination of the DNA with protein must be avoided to eliminate misleading results. The technique of DNA covalent-binding studies is

therefore critical, and this aspect has been recently reviewed (Bolt and Laib,1980). Isolation and chemical identification of the DNA alkylation products may be the key to arrive at meaningful and well-founded conclusions. In an extensive literature survey Lutz (1979) indicated that there is some degree of correlation between the hepatocarcinogenicity of a compound and covalent DNA binding in vivo.

Another aspect of great importance is selection of the dose to be administered. This can be influenced by the extent of alkylation expected. Selection of the dose is facilated by a knowledge of the pharmacokinetics of the compound, but dose-extrapolation is not possible in all cases. For example, the pharmacokinetic parameters of the carcinogenic trans-4-dimethylaminostilbene remain constant over a dose range of 6 orders of magnitude (Neumann, 1979), and other carcinogens often reveal non-linear pharmacokinetics (see chapter B 2.3.1).

A major technical problem of DNA binding assays is the incorporation of radioactivity derived from the compound administered into the 1-carbon or 2-carbon pools by routes of intermediary metabolism. As the progression of radioactivity with time may be different in the natural bases (increasing after dosing) and alkylation products (showing action of repairing enzymes), the choice of time of sacrifice is very important - see discussion by Lutz, (1979) who considered species, strain and age as important variables. It should be emphasised that counting only the radioactivity of the isolated DNA may lead to misinterpretations and should be avoided.Characterisation of the alkylation products should be attempted in order to avoid false interpretations.

When all these methodological pitfalls are considered, it is not surprising that qualitatively divergent results have been obtained from different laboratories. Standardization of covalent DNA-binding assays is a prerequisite for obtaining comparable results. For a quantitative comparison of different data it must be borne in mind that the "covalent binding index" introduced by Lutz (1979), expressing the relation between DNA damage and dose administered, is time-dependent because of the existence of repair and metabolic processes .
It is clear that at present DNA-binding assays are not routine procedures. They are invaluable, however, for in-depth research which could follow the standard battery of tests and may also contribute to an understanding, on a biochemical basis, of the species differences observed

in carcinogenicity studies. Hence, today, studies on covalent DNA binding contribute to a thorough examination of those compounds which are recognised as possible carcinogens, especially when one must distinguish between "genotoxic" and "non-genotoxic" mechanisms .

Negative results in DNA-binding and mutagenicity studies should focus attention on other aspects, such as liver growth, DNA synthesis and cell proliferation.

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